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14. ABSTRACT
Our research efforts during the last funding year have been focused on optimization of designs and experimental conditions for molecular beacons. Specificity of molecular beacons (MBs) is affected by length and characteristics of the loop and stem sequences and efficiency of the quencher molecules. Although we have shown specificity of MBs for survivin and cyclin D1 mRNA, recent advances allow us to make MBs with even higher signal to noise ratio. We have selected new targeting sequences in survivin and Her-2/neu mRNA for MB sequences and used a new Black Hole Quencher molecule. In order to increase specificity and sensitivity of detection of breast cancer cells, we developed nanoparticle fluorescence imaging probes, called quantum dots (QDs), to detect the expression of EGFR proteins, which are highly expressed in many breast cancer cells. QDs targeting to survivin, Cyclin D1 and Her-2/neu are under developing in our laboratory. A major progress is to obtain an approval of our human protocol (Phase I) from the Human Investigation Committee of the DOD. We are ready to start the human trial in breast cancer patients. Additionally, our manuscript was published in March 2005 issue of Cancer Research.

15. SUBJECT TERMS Breast tumor markers, Her-2/neu, Cyclin D, survivin, EGFR, uPAR, molecular beacons, molecular imaging of cancer cells, Early detection of breast cancer, identification of ductal carcinoma *in situ,* ductal lavage, Fine needle aspiration, women at high risk of breast cancer

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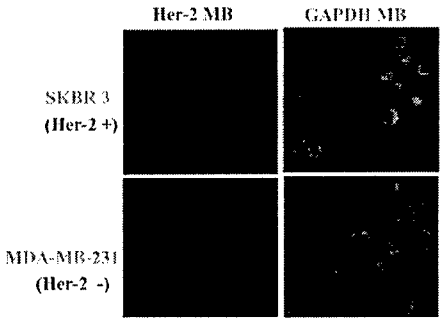
Introduction:

The objective of our study is to detect breast cancer at early stage and increase survival rate of breast cancer patients. Our research supported by this DOD Idea Award is to use molecular beacon (MB) probes to detect breast cancer cells expressing tumor marker genes. Although we have developed MB probes specific for survivin, cyclin D1 and Her-2/neu mRNA, we will examine the protein levels of above tumor markers simultaneously to increase the specificity and sensitivity of the detection. Furthermore, we have added another tumor marker EGFR, which is highly expressed in many breast cancer cells (1). During the last funding year, we have been working on optimizing MB design and experimental conditions, developing nanoparticle fluorescence probes for tumor markers and obtaining an approval of our human protocol from the Human Investigation Committee of the DOD.

Body of the progress report

1. Determination of specificity of Her-2/Neu MB in breast cancer cell lines.

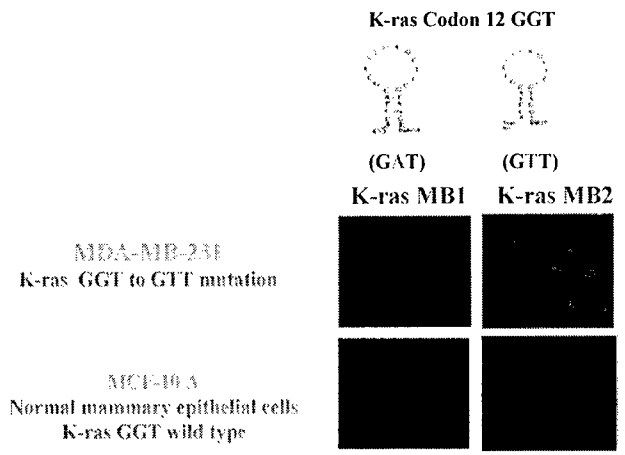


Results from our previous study have shown that survivin and cyclin D 1 MBs produced fluorescence signals in breast cancer cell lines expressing those tumor marker genes. To determine the specificity of Her-2 MB, we used Her-2 + (SKBr-3) and Her-2 – (MDA-MB-231) breast cancer cell lines and found that incubation of Her-2 MB with breast cancer cells produced fluorescence signal in SKBr-3 cell (blue) but not in MDA-MB-231 cells, suggesting Her-2/Neu MB is highly specific for Her-2/Neu mRNA. We also found that use of blue fluorescence dye for Her-2/Neu MB has some limitations such as a weaker fluorescence signal and unable to use our confocal microscope. Therefore, a new Her-2/neu MB with Cy5 dye was synthesized.

2. Detection of a mutant oncogene, K-ras, in breast cancer cells using K-ras MB.

The presence of point mutations in K-ras gene is found in many human tumor types (2). K-ras mutation is also detected in breast cancer tissues, although the percentage of K-ras mutations is not as high as other tumor types (3, 4). A major advantage of the stem-loop probes is that they can recognize their targets with a higher specificity than the linear probes. Properly designed MBs

could discriminate between targets that differ by as little as a single nucleotide (5). We have designed MB probes specific for several common K-ras point mutations and found those K-ras MBs were able to differentiate K-ras mRNA containing a single base pair mutation (6). As shown in the figure, breast cancer cell line MDA-MB-231, which has a K-ras GGT to GTT mutation, displays red fluorescence after incubating with K-ras MB2 (GTT) but not K-ras MB1 (GAT). On the other hand, normal human mammary epithelial cell line MCF-10 A does not have fluorescence signal after incubating with either K-ras MB1 or K-ras MB2. This result suggests that K-ras MBs can also be used for the detection of breast cancer cells. However, unlike pancreatic cancer cells with 95% of them harboring K-ras mutation at codon 12, mutations of k-ras in breast cancer cells are in several locations, which potentially affect the sensitivity of cancer cell detection since MBs for each K-ras mutation have to be used for patients' samples with unknown status of K-ras mutation.



Detection of mutant K-ras mRNA in breast

3. Re-designing MBs for survivin and Her-2/Neu MB probes.

Previously, we used 4-({4'-(dimethylamino)phenyl]azo)benzoic acid (Dabcyl) as a quencher molecule, which was the best available quencher. Recently, a new class of quencher molecules, called black hole quencher (BHQ), has been developed by Biosearch Technologies. Dabcyl has inadequate absorption that overlaps very poorly with fluorescent dyes emitting above 480 nM. BHQ dyes have much larger signal-to-noise ratio, which will greatly reduce background fluorescence signals. In addition, we further examined cDNA sequences of survivin and Her-2/Neu genes and have identified better oligonucleotide sequences for survivin and Her-2/Neu MBs as shown in Table-1. Further, we also designed a negative control MB containing scrambled DNA sequence that does not share homology with any known gene sequences. All MBs have been synthesized and are ready to use for our study.

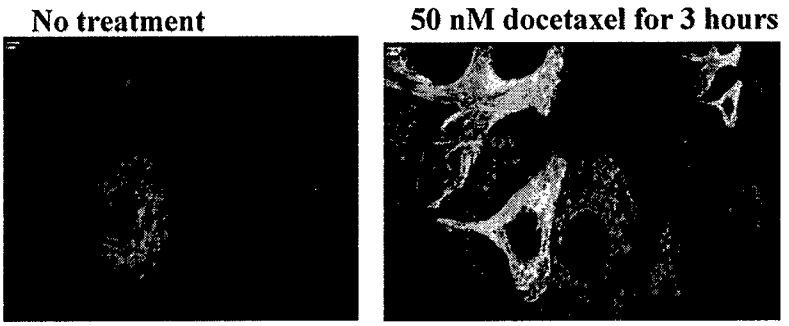
Table-1 Design and oligonucleotide sequence of molecular beacons.

Re-designed MBs	MB sequences
Survivin-MB #5	5'-Cy3-TGGCTCTTTCTCTGTCCAGAGCCA-BHQ2-3'
Her-2/Neu MB #2	5'-Cy5-ACAGGAGTGGGTGCAGTTG CCTGT-BHQ2-3'
Scrambled MB	5'-Cy3-AGACGCGCATCACATTCTC CGTCT-BHQ2-3'

4. MBs can be used to monitor the level of gene expression and location of specific mRNA in living cells real time.

Previously, we reported that MBs can be transfected into viable cells and that fluorescence intensity produced inside the cells correlated well with the changes of the level of gene expression. In collaboration with Dr. Paraskevi Giannakakou, MCF-7 breast cancer cells stably transfected with a GFP-tubulin fusion gene were transfected with Survivin MB (Cy-3) and then cultured in the presence or absence of 50 nM doxetaxel, at which docetaxel concentration induces upregulation of survivin expression. The cells were examined using a confocal microscope with a live-imaging system. First, we observed that red fluorescence signals were co-localized to green microtubules, suggesting that survivin mRNAs are located around microtubules. Interestedly, docetaxel-treatment stabilizes microtubulin resulting formation of bundles of green microtubules. However, in cancer cells with a high level of survivin, as indicated as red clusters of fluorescence dots, few green microtubule bundles were found. It is possible that upregulation of survivin gene expression in those cells prevents the effect of docetaxel even before activation of the apoptotic pathway.

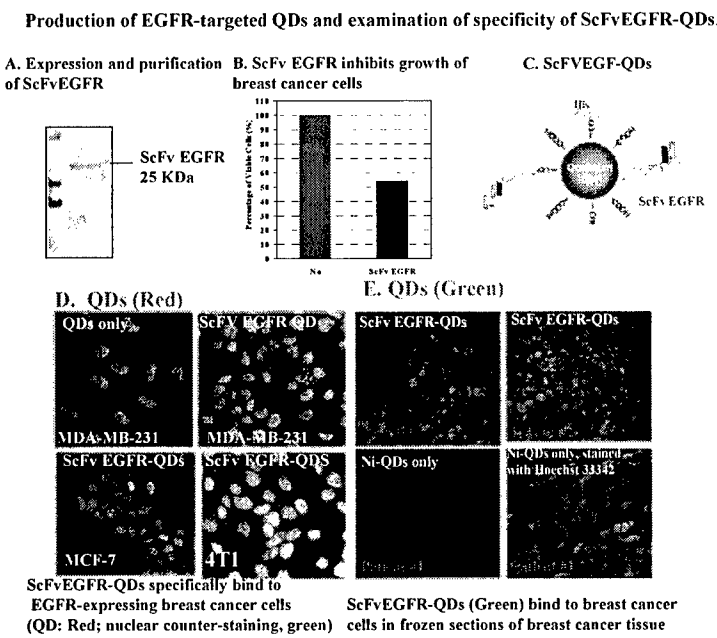
Real-time detection of the level of location of survivin mRNA in living cells



5. Engineering fluorescence quantum dot nanoparticles targeting to EGFR.

It is well known that human tumor contains heterogeneous cell population expressing different biomarkers. Recently, our lab has developed a novel approach to conjugate tumor-targeted peptides to fluorescence nanoparticles, quantum dots. Emerging as a new class of fluorescent probes for *in vivo* biomolecular and cellular imaging, these quantum dots (QDs) are tiny,

nanometer-scale light-emitting particles. In comparison with organic dyes and fluorescent proteins, quantum dots have unique optical and electronic properties such as size-tunable light emission, improved signal brightness, resistance against photobleaching, and ability to simultaneous excite multiple fluorescence colors (7). These properties are most promising for improving the sensitivity of molecular imaging and quantitative cellular analysis by 1-2 orders of magnitude. Another advantage is that multicolor QD probes can be used to image and track multiple tumor markers simultaneous, which will most likely increase the specificity and sensitivity of cancer detection. We have also produced a single chain anti-EGFR antibody (ScFvEGFR) in a bacteria-expressing system. As shown in the figure, ScFvEGFR is conjugated to QDs through Ni-NTA, which binds to 6 x his tag in the C-terminal of the single chain antibody. We showed that those ScFvEGFR-QDs specifically bind to breast cancer cells expressing a high level of EGFR both in culture and in frozen tissue section of breast cancer tissue. We are also in the process of production of survivin, cyclin D1, Her-2/neu and HIF-1 alpha antibody-conjugated QDs. HIF-1 alpha is a hypoxia-inducible transcription factor that is upregulated in breast cancer tissues (8). We will use different colors for MB and QDs probes. Therefore, the levels of survivin, cyclin D1 and Her-2/Neu mRNA and protein levels of above tumor markers as well as EGFR and HIF-1 alpha can be measured in a single cell level, which should enhance the sensitivity of cancer cell detection.

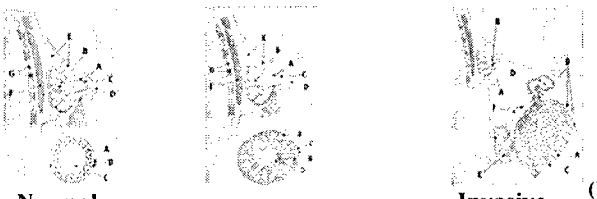


6. Strategies for conducting a clinical trial on the detection of breast cancer cells using molecular beacons.

At present, we have obtained approval from both Emory IRB and the Human Investigation committee of DOD to perform a phase I clinical trial. As shown in the figure, this phase I trial is to evaluate the feasibility of detecting breast cancer cells in fine needle biopsy or ductal lavage

samples using molecular beacons targeting survivin, cyclin D1 and Her-2/neu mRNA. We propose that on the same sample and in addition to MB detection of mRNA, we will use EGFR, survivin, cyclin D1 and HIF-1 alpha antibody conjugated QDs to determine the levels of those biomarkers. Please see the following figures for detailed information on the procedure.

Human clinical trial design:

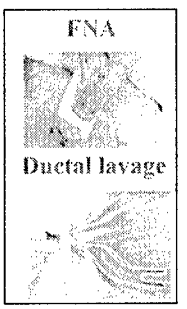


Normal DCIS Invasive (Breastcancer.org)

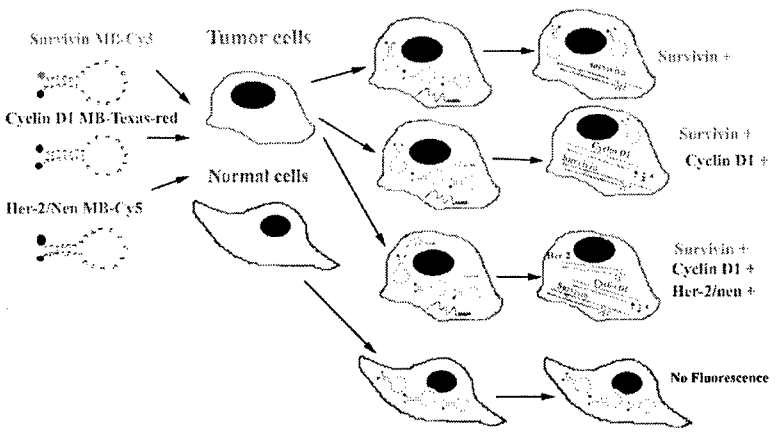
Two phases:

Phase I. Feasibility of detection of breast cancer cells in fine needle aspiration and ductal lavage samples obtained From breast cancer patients.
Tumor markers: survivin, Her-2/neu and cyclin D1.

Phase II. Determination of the correlation between detection of tumor marker gene expressing cells in fine needle aspiration or ductal lavage and the presence of early stage of breast cancer in women at a high risk for developing breast cancer.



Detection of breast cancer cells expressing one or more than one tumor marker using molecular beacons



Key Research Accomplishments:

1). We have demonstrated specificity of all three MBs in detection of corresponding mRNAs.

2). We have improved our MB design using more advanced quencher molecules and better cDNA sequence region of the genes.

3). We have developed fluorescence QD probes for detection of protein levels of the tumor marker genes.

4). We have obtained approval from the Human Investigation Committee of the DOD to conduct Phase I study (A full human protocol and consent form are attached in the Appendix). Since molecular beacon is classified as nanotechnology by NIH/NCI, we believe that our clinical trial is the first to apply nanoprobes for breast cancer detection.

5). We have published a manuscript entitled "Real-Time Detection of Gene Expression in Cancer Cells using Molecular Beacon Imaging: New Strategies and Implications for Cancer Research" in March 15th 2005 issue of Cancer Research (attached in the Appendix).

6). We have filed two provisional patent applications:

U.S. Provisional Patent, filed in December, 2004,

Title: Methods and applications of molecular beacon image for cancer cell detection.

U.S. Provisional Patent, filed on May 2, 2005 (# 604-676812).

Title: Multifunctional nanoparticles for non-invasive *in vivo* imaging and treatment of human cancer.

7). We have produced promising results on tumor targeted nanoparticles for detection of breast cancer. Those results were used as preliminary results to apply for the Cancer Center of Nanotechnology of excellence (CCNE) in March 2005. Currently, this application is recommended for funding by NIH/NCI.

8). We have also developed tumor-targeted magnetic iron oxide nanoparticles for non-invasive imaging and treatment of breast cancer. Based on our preliminary results, we have submitted a DOD Idea Award Application entitled "Development of Targeted Nanoparticles for Non-invasive *in vivo* Imaging and Treatment of Breast Cancer" in May 2005.

Reportable Outcomes

We have optimized design and experimental conditions for MBs. We have also developed new nanotechnology that uses tumor-targeted nanoparticles for breast cancer detection. We have obtained an approval of the Human Investigation Committee of DOD for conducting the Phase I clinical trial in breast cancer patients. Success in the Phase I study will lead us to further use this approach for early detection of breast cancer in high risk population.

The goal of next funding year is to conduct clinical trial to determine the feasibility of detection of the breast cancer cells in clinical samples from breast cancer patients using a combination of MBs and QDs.

Conclusions

In summary, we have optimized our experimental conditions for detection of breast cancer cells using the MB-fluorescence imaging technology. We have developed fluorescence QD nanoprobes to detect breast cancer cell through examination of protein levels of tumor markers. We are ready to start clinical trial and have developed detailed protocol for this trial.

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Appendix

Research Article: Cancer Research, 2005

Human protocol

Consent form

Protocol title:
Early Detection of Breast Cancer Using Molecular Beacons

Subtitle:
Examination of the Sensitivity of Using Molecular Beacons Targeting Survivin, Cyclin D1 and Her-2/neu mRNAs to Detect Breast Cancer Cells in Ductal Lavage and Aspirates of Fine Needle Biopsy in Breast Cancer Patients

Principal Investigator:
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Sponsor's Name: Department of Defense

Phase of the Study: Feasibility

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Introduction

Breast cancer is the most common type of cancer and one of the leading causes of death among women. A crucial factor to increase survival is to diagnose it early. Although early screening with mammography decreases the mortality of the disease, nearly 20% of breast cancer patients are still missed by mammography. Furthermore, of all patients with abnormal mammograms, only 10 to 20% were confirmed to be breast cancer by biopsy [1]. At present, there is no reliable serum tumor marker for diagnosis of breast cancer. Therefore, development of novel approaches for early diagnosis of breast cancer is of critical importance for the successful treatment and for increasing survival of the patients. It is well known that over 90% of breast cancers arise in the epithelial cells lining the ductal system. Most breast cancers developed over a period of 8 to 10 years before detected by standard methods. In order to detect early stage breast cancer or ductal carcinoma *in situ* (DCIS), it is important to develop novel and non-invasive approaches with high sensitivity and specificity.

It is well established that cancer cells develop due to genetic alterations in oncogenes and tumor suppressor genes and abnormalities in gene expression that provide growth advantage and metastatic potential to the cells. A novel way of achieving early detection of cancer is to identify the cancer cells through detection of mRNA transcripts that are expressed in the cancer cells but at low levels or not expressed in normal cells. We have developed a molecular beacon (MB)-based approach for direct examination of gene expression in viable and fixed cells[2, 3]. The proposed study focuses on the evaluation of the feasibility of detection of breast cancer cells in ductal lavage and/or fine needle aspiration using MBs targeting survivin, cyclin D 1 and Her-2/neu mRNAs, which are tumor markers found in over 70 to 80% of DCIS tissue samples.

Our working hypothesis is that simultaneous detection of several tumor markers for breast cancer using MBs has a high specificity and sensitivity in detecting cancer cells. The ultimate goal is to develop a simple, sensitive and efficient clinical procedure for early detection of breast cancers.

MBs are oligonucleotides with a stem-loop hairpin structure, dual-labeled with a fluorophore at one end and a quencher at the other. Delivering MBs into cells produce fluorescence signal if the MBs hybridize to target mRNAs. Thus, when the target mRNAs correspond to the molecular markers of a cancer, cancer cells (bright) can be distinguished from normal cells (dark)[2]. We have established the structure-function relationships of MBs and experimental conditions that enable us to identify breast cancer cells using MBs.

Significance

The proposed study will be the first to apply state-of–the art MB-based methodology for detection of breast cancer. Survivin is a newly discovered protein in the inhibitor of apoptosis protein family (IAP). This investigation will also be the first such study to evaluate the feasibility of using survivin as an early breast cancer marker. Since MB is highly specific in detecting target mRNAs, and MBs targeting various genes can be labeled with different fluorescent-dye molecules and delivered into single cells, expression of several tumor marker genes in a single cell can be analyzed at the same time. Human breast cancers contain heterogeneous cell populations with various genetic changes. Simultaneous detection of overexpression of several tumor marker genes, especially when a single cell expresses more than one marker gene, may have a high predictive value for identifying cancer cells, and therefore increase the sensitivity and specificity of cancer detection. For example, although survivin is detected in over 70% of breast cancer tissues, about 30% of patients don't express survivin and will be missed if only survivin MB will be used. About 20 to 30% of DCIS tissues are also negative for cyclin D or Her-2/neu. We plan to use MBs targeting survivin, cyclin D1 or Her-2/neu mRNAs that are

labeled with different fluorescence dye molecules to determine the relationship between the detection of the cells expressing none, one, two or three markers and the feasibility of early detection of breast cancers in ductal lavage or fine needle aspirates. The results of our study *in vitro* in human breast normal and cancer cell lines have shown that delivery of a mixture of survivin and cyclin D1 MBs into fixed cells produced fluorescent signals in breast cancer cells but not in normal breast cells. Interestingly, the fluorescence intensities in the cells correlated well with the level of the gene expression in different tumor cell lines. Previous methods for detecting gene expression *in situ* were not quantitative since the signals were amplified by either the presence of multiple fluorescent-dye labeled nucleotides in an oligonucleotide probe or amplification of the signals with secondary antibodies to labeled nucleotides. Since each MB has only one fluorophore and unbound MBs do not fluoresce, the fluorescence intensity generated by hybridization of the MB with a specific mRNA should reflect more accurately the level of the mRNA expressed in the cells. Additionally, since only a small amount of abnormal cells are present in a large amount of normal cell background in clinical samples, there is a clear advantage of direct fluorescence imaging of individual cells expressing tumor marker genes for early detection of cancer cells compared to conventional RT-PCR to amplify the expression of tumor marker genes from isolated total RNA, which may be difficult to detect the differences in the level of gene expression in a few cancer cells over the normal background.

Therefore, the MB–based cancer cell detection has the potential to become a simple clinical procedure for early detection of breast cancer with a high sensitivity and specificity.

Experimental procedures:

Examination of the sensitivity of using molecular beacons targeting survivin, cyclin D 1 and Her-2/neu mRNAs to detect breast cancer cells in ductal lavage and aspirates of fine needle biopsy in breast cancer patients.

First, we will examine the specificity and sensitivity of MB-imaging cancer cells in ductal lavage or fine needle aspiration from breast cancer patients. This study will allow us to determine whether the MB is more specific and sensitive than cytological method in detecting breast cancer cells. The proposed study will also provide us with new information regarding using survivin, cyclin D 1 and Her-2/neu as tumor markers for identifying the breast cancer cells at DCIS stage.

Study subject selection:

Eligibility Criteria:

Patients of all ages and races diagnosed with breast cancers or with possibility of breast cancer, who

come to Emory Clinic or Emory Hospital and are able to read and speak English.

Ineligibility Criteria:

Breast cancer patients who have received chemotherapy or radiation therapy for breast cancer or other cancers within last three months, or are currently pregnant or nursing.

Recruitment methods and the informed consent process:

Breast cancer patients, who come to Emory Clinic or Emory Hospital and are cared for by Co-PIs, Drs. Wood and Carlson, in the Departments of Surgery and the Winship Cancer Institute, will be informed by their physicians about the clinical trial. If the patients are interested in participating in the

study, the informed consent forms will be given to the patients. The patients and their family members will allow time to read and ask questions about the study. If the patients are unable to provide their own consent to participate the study, their legally authorized representative will be contacted. The informed consent form will be given to the representative and allow time to read and ask questions. The patients will only be enrolled in the study after obtaining signed informed consent form from their legally authorized representatives.

If subjects are willing to participate in the study and the consent form is signed, extra samples of fine needle aspiration from a scheduled diagnosis procedure will be collected for the MB study. Additionally, ductal lavage will be performed right before the scheduled operation for removing the breast tumor under general anesthesia according to the procedures described in Experimental Design and Methods.

All study procedures will be conducted in compliance with the protocol, GCP and the applicable regulatory requirements.

Study protocol:

The purpose of this study is to determine whether survivin, cyclin D 1 and Her-2/neu MBs are able to identify breast cancer cells in fine needle aspirate (FNA) and ductal lavage samples which have a high probability of the presence of breast cancer cells. We will also determine the percentage of the cells that express one, two or three tumor marker genes at different stages of the disease, especially in the samples from patients diagnosed with DCIS.

Fine needle aspiration:

Fine needle aspiration is the easiest and fastest method of obtaining a breast biopsy. It is an FDA approved biopsy procedure and has been used in outpatient clinics routinely as a diagnosis procedure for breast cancer [6]. It uses a thin needle on a syringe to draw fluid and/or cellular material from breast tissues.

FNA samples will be collected during a scheduled diagnostic procedure by Dr. Lewis in the outpatient exam rooms in the Winship Cancer Institute at Emory University, Surgical Oncology Suite, Clinic C, 1365 C Clifton Road, NE, Atlanta, GA. FNA is performed under local anesthesia. Under sterile conditions, a fine hollow needle that is attached to a syringe to extract fluid from a solid lesion is inserted into the breast mass of breast cancer patients. The needle used in this procedure is very small (smaller than those used to draw blood). The procedure takes a few minutes. The FNA samples will be sent to Dr. Yang's research laboratory immediately to determine whether tumor marker-gene expressing cells are present in the FNA samples.

Recovery after the FNA procedure is generally quick and uncomplicated. Most patients are able to resume normal activity almost immediately afterwards. Pain is minimal and can usually be managed with an over-the-counter pain reliever. Complications for procedures are rare, but excessive swelling, redness, and bleeding or other drainage can indicate an infection or abnormal bleeding. The PI and CO-PIs, who usually are the physicians for these patients, should be notified immediately.

Ductal lavage:

Ductal lavage is an FDA-approved and minimally invasive procedure to collect breast ductal epithelial cells for cytopathological analysis[4, 5]. This procedure is typically performed in an outpatient exam room in a clinic and takes about 30 minutes. However, for patients undergoing lumpectomy or more extensive surgery, ductal lavage will be performed by Dr. Wood or Dr. Carlson in the operating room of the Emory University Hospital after administration of general anesthesia and before surgery. Credentials for the CO-PIs to perform the procedures are stated in Appendix A.

In the operating room and under sterile conditions and general anesthesia, a microcatheter (Firstcyte ultraslim dilator, an FDA-approved Class II device from Cytyc Health Corp., MA), will be inserted 0.5 to 1.0 cm into a nipple orifice and 10 ml of sterile saline will be slowly infused. For each patient, two ducts will be lavaged. The effluent fluid from each duct will be collected and placed in separate vials that are labeled with a specific study ID number for each patient. The samples will be placed on ice and sent to Dr. Yang's research laboratory immediately to determine the status of expression of three tumor marker genes in the cellular fraction of the duct lavage using a mixture of survivin, cyclin D1 and Her-2/neu MBs. About 13,500 cells per duct can be collected for analysis of the presence of normal, atypical, or malignant breast ductal cells. The procedure for ductal lavage under general anesthesia can be finished within 10 minutes, which will prolong the general anesthesia time for 10 minutes and may slightly increase the risk for the patients.

For each breast cancer patient, we plan to collect three samples for this study: 1) FNA samples from a scheduled diagnostic procedure, 2) ductal lavage before surgery, and 3) breast cancer tissues after surgery. After examination of the FNA and ductal lavage samples from breast cancer patients, we should be able to determine which method is more sensitivity for detection of breast cancer cells.

Cellular analysis of tumor marker gene expression using molecular beacons

The ductal lavage and FNA samples will be processed immediately after collection to ensure the quality of RNA in the cells. After a brief centrifugation, the cell pellets will be placed in pretreated glass slides using a cytospin. About 10 to 15 cytospin slides will be obtained from one ductal lavage. After fixing the cells in ice-cold acetone, the slides will be incubated with a mixture of survivin, cyclin D1 and Her-2/neu MBs at optimized incubation conditions for 1 hour and then examined under a confocal microscope.

Breast cancer cell lines with known levels of expression of survivin, cyclin D1 and Her-2/neu genes will be used as positive controls. Normal human mammary epithelial cell lines and human primary fibroblasts will also be used as negative controls. Images of cells from patient sample, positive and negative control will be taken using the identical instrumental sittings for confocal mincroscope. The levels of fluorescent intensity for each fluorescent dye will be analyzed on all of the cells observed on the slides. The cells displaying a fluorescent intensity that is two fold-higher than negative control cells will be labeled as positive cells. In comparison with the fluorescence intensity in breast cancer cell lines, we should be able to score the fluorescent intensity in patient's samples from -, +, ++, +++ to ++++. The number of cells showing positive labeling for each MBs and the number of cells have one, two or three gene expression will also be recorded. We will further compare results obtained from FNA and ductal lavage from the same patients to determine the specificity and sensitivity of detecting cancer cells.

Since MBs for each gene are labeled with different fluorescent dyes, the number of the cells overexpressing survivin, cyclin D 1, Her-2/neu, two of the genes or all three genes in ductal lavage and FNA samples will be determined. We will also compare results obtained from FNA and ductal lavage to determine the specificity and sensitivity of detecting cancer cells. Since FNA samples may contain more cancer cells, this will also provide with us an additional source to evaluate the specificity of the MB-detection. Upon finishing the examination, the same slides will be stained with H&E and analyzed by a cytopathologist (Dr. Lewis) for the presence of benign, atypical or malignant cells. We will then compare the results of the MB detection with cytological findings and pathological diagnosis after surgery to determine whether MB method is more sensitive and specific for the detection of breast cancer cells than current cytology methods. We will also determine if the MB-detection of the cells expressing survivin, cyclin D 1 and Her-2/neu genes is able to identify cancer cells at DCIS

stage. For each patient, we will obtain breast cancer and surrounding normal tissues after surgery and examine for the expression of survivin, cyclin D 1 and Her-2/neu on frozen tissue sections by immuostaining with specific antibodies.

Current method for identification of different cell types in ductal lavage and FNA samples is by morphological classification. Development of molecular approaches for the detection of cells at different stages of the tumorigenesis will enhance the specificity and sensitivity for early detection of breast cancer cells. Since each MB targeting a specific mRNA is labeled with a specific fluorophore, a major advantage of the MB-based approach as compared with RT-PCR and immunohistochemistry for early cancer detection is that a mixture of MBs targeting multiple tumor specific mRNAs can be delivered to a single cell at the same time, and the expression of all these markers can be observed in a single assay using a fluorescence microscope.

Adverse effect /IND safety report:

Fine needle biopsy is a minimally invasive procedure that has been used in clinic routinely for diagnosis of breast cancer. Ductal lavage is also a minimally invasive procedure and has been used on over 500 high risk women and no serious adverse effects were found [4].

For breast cancer patients, ductal lavage will be collected during the surgery and fine needle aspirates will be obtained from scheduled biopsy. Participation in the study will not add to significant risks or discomforts to the patients. It is possible that ductal lavage prolongs anesthesia time of the patients for 10 minutes, which may potentially increase risk for the patients. Close monitoring of the patients during general anesthesia, as it done routinely, should reduce the risk of any adverse effects related to ductal lavage.

Any adverse effects related to participation in the study will be documented whether or not considered to be related to the study. This definition includes inter-current illnesses and injuries and exacerbation of pre-existing conditions. The following information will be included in the IND safety reports: subject identification number and initials; investigator's name and name of the medical treatment facility/hospital or research facility; subject's date of birth, gender, and ethnicity; date of procedure performed; signs/symptoms and severity; date of onset; date of resolution; relationship to the study; action taken; concomitant medications including dose, route and duration, and date of the last dose.

Unanticipated problems involving risk to volunteers or others, serious adverse events related to participation in the study and all volunteer deaths should be promptly reported by phone (301-619-2165), by e-mail (hsrrb@det.amedd.army.mil), or by facsimile (301-619-7803) to the Army Surgeon General's Human Subjects Research Review Board. A complete written report should follow the initial telephone call. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Material Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

In case of adverse effects related to the study, the PI and/or CO-PI will be notified immediately. The PI will refer the patients to Army Hospitals in Georgia, to receive treatment free of charge for injuries directly caused by the study. The primary physician and the PI of the study will follow up the patients until they are fully recovered. The Amy will not pay for transportation to and from the hospital

or clinic If the patients paid our-of pocket expenses for medical care elsewhere for injuries caused by this study, they will need to contact the PI. All questions concerning the medical care will be addressed by the PI and CO-PIs. If there are issues cannot be resolved, the U. S. Army Medical Research and Material Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at 301-619-7663/2221 will be contacted.

Proposed patient sample size:

The purpose of this protocol is to carry out a pilot study for the determination of sensitivity and specificity of detection of breast cancer cells using the molecular beacon approach. We plan to use a minimum sample size that will give us information on the feasibility of detection of breast cancer cells in clinical samples. We plan to analyze samples from 50 breast cancer patients, which include at least 25 patients with DCIS. However, we will use statistical analysis such as power calculations based on results from our pilot study, which will be obtained after we start the trial, to determine the final sample size. The informed consent forms will be signed by the cancer patients before performing any procedures.

A total of 50 breast cancer patients will be enrolled in this study.

Statistical methods for data analysis

Sensitivity and specificity of the MB-based approach in ductal lavage will be analyzed using standard statistical analyses such as student's t-test or Mann-Whitney test. Differences in the prevalence of detecting cancer cells in ductal lavage or FNA from breast cancer patients by the MB detection and by cytology will be calculated by a 2-tailed chi-square test. The prevalence of identifying tumor cells by MB-detection of the cells expressing each gene alone, or more than one gene will be calculated by Chi-square test or Kruskal-Wallis one-way analysis of variance on ranks. A P-value of < 0.05 will be considered statistically significant.

Data Handling:

All patients and normal control subjects enrolled in the study will be assigned an ID number by the PI. The clinical samples will be collected by Dr. Wood, Dr. Carlson, and Dr. Lewis, who are co-investigators of the protocol. All samples will be placed in sterile tubes pre-labeled with the patient's ID number and sent to Dr. Yang's research laboratory immediately. Only the PI and CO-PIs will have an access to the names and hospital record numbers of the participating breast cancer patients. The research staff in the lab will only be given the ID number of each sample. All research results will be recorded using the ID numbers. The original experimental records and CDs for storing the image files will be kept in Dr. Yang's laboratory and office in locked cabinets located in the Clinic C Building, Room C-4088 and C-4038, 1365 C Clifton Road NE, Atlanta Georgia. Information on study ID numbers, patient's names and hospital numbers will only be kept in Dr. Yang's computer in her office and with a copy of CD disk locked in the cabinet. All research results will be stored and kept in Dr. Yang's office for three years or until the study and all follow-up studies are finished. The paper records concerning the patient's information, hospital number and study ID number will be shredded using a paper shredder. The files in computer hard driver and on CDs will be deleted.

The PI will inform the CO-PIs about the experimental results from all participants. Since the results are non-CLIA approved research results, the participants will not be informed about the test results.

People other than those doing the study may look at both medical charts and study records. Agencies that make rules and policy about how research is done have the right to review these records. These include the Department of Defense, the National Cancer Institute, the Emory Clinic, Emory University Hospital and the Emory University Institutional Review Board. Records can also be opened by court order. We will use a study ID number rather than patient's name on study records where we can. Patients' name and other facts that might point to the patient or normal subjects will not appear when the results of this study are presented or published.

Modification of the study protocol:

Any change in the protocol, personal, and numbers of enrollment will be submitted to Emory IRB and the HSRRB of the DOD for an approval.

Requests for termination or extension of the protocol will also be submitted to Emory IRB and the the HSRRB of the DOD for an approval.

In the events of a departure from the protocol, a subject withdraws from the protocol and termination of a participant by the PI and/or CO-PIs, the PI will be notified and reasons for subject withdraw or termination will be recorded. The PI will report all information on the continuation application form to Emory IRB each year and in the annual report to DOD Breast Cancer Research Program.

The subject's participation may be terminated by the PI and Co-PIs if she becomes pregnant before the procedure, has received chemotherapy or radiotherapy, or have developed other illnesses that are inappropriate for conducting ductal lavage or FNA procedure.

Timeline for the study

We plan to start this sub-project in June 2005 and expect to finish by June 2006.

Appendix A

Qualification of the PI and Co-PIs for the study

Principal investigator: Lily Yang, MD. PhD, is an Assistant Professor in the Department of Surgery and the Winship Cancer Institute. Dr. Yang has been working in the field of cancer research since 1986 and is trained in both cellular and molecular biology. Her PhD. study involved the cellular origin of liver cancer and identification of liver stem cells. She worked on projects for the development of new approaches for cancer gene therapy including preclinical studies on the delivery of angiogenic inhibitors by adenoviral vectors for the treatment of primary and metastatic breast cancers. Dr Yang has been working on targeting survivin as a therapeutic approache for breast cancer since 1999. In May of 2001, she started working on molecular imaging of breast and pancreatic cancer cells using MBs detecting mutant K-ras, survivin, cyclin D1 and Her-2/neu. Many assays and methods for evaluation of the specificity of MBs have been established in her laboratory. She will direct all aspects of the project and is responsible for experimental design, protocol, data analysis and manuscript preparation.

William C. Wood, MD, is the Joseph Brown Whitehead Professor and the Chairman of the Department of Surgery at Emory University. Dr. Wood is a world-leading expert in breast cancer treatment. He will perform the procedures for ductal lavage on breast cancer patients and will also provide us with surgically resected human breast cancer and adjacent normal tissues. He will participate in study design for the detection of cancer cells and perform ductal lavage of breast cancer patients, the high risk population and normal volunteers.

Grant W. Carlson, MD, Dr. Carlson is Professor of Surgical Oncology in the Department of Surgery at Emory University. Dr. Carlson's has expertise in both breast cancer surgery and plastic surgery for breast remolding. Dr. Carlson participated in a multi-center research project on examination of cell types in ductal lavage of high risk women as an indication for tamoxifen risk-reduction therapy and has performed ductal lavage procedure. He will be responsible for performing ductal lavage in breast cancer patients, normal volunteers and women with high risk of developing breast cancer.

Melinda M. Lewis, MD., FCAP: Dr. Lewis is an Associate Professor, Director of Cytopathology in the Department of Anatomic Pathology and Director of Fellowship for Cytopathology. She is an expert on breast cytology and is a board-certified cytopathologist for performing FNA and characterization of cell types in FNA and ductal lavage samples. She has been responsible for cytopathology in Breast Cancer Clinic in the Winship Cancer Institute at Emory University and performed FNA daily on the breast cancer patients since 1988. Her research interests focus on the application of immunohistochemical and molecular techniques to cytologic specimens to enhance diagnostic and prognostic information. One of her research projects is on the comparison of immunohistochemistry and fluorescence *in situ* hybridization in the evaluation of Her-2/neu in image-guided breast fine needle aspiration. Dr. Lewis is also involved in a research project on the examination of cell types in ductal lavage of women with high risk of breast cancer for preventive tamoxifen treatment. In the proposed study, Dr. Lewis will be responsible for performing FNA and cytological analysis of cell types in ductal lavage and FNA samples.

Xiang-Hong Peng, MD., Postdoctoral Fellow: Dr. Peng is a medical oncologist with research training in molecular and cellular biology. He will be responsible for characterization of MBs, cellular assays, analysis of ductal lavage and FNA samples and molecular imaging of cancer cells.

Dr. Yang (PI) has filled a patent application, U.S. patent, 60/439,771, on January 13, 2004, entitled "Methods of detecting gene expression in normal and cancerous cells". The application is pending and under reviewing by the patent office.

Co-PIs don't have a conflict of interest for this study.

Appendix B

Medical Mediator

Ruth O'Regan, MB, MRCPI, MB FRK

Dr. Ruth O'Regan will be the medical monitor for this study. Dr. O'Regan is an Assistant Professor of Hematology and Oncology; Director, Translational Breast Cancer Research Program. Dr. O'Regan joins the WCI from Northwestern University in Chicago where she was an Assistant Professor of medicine at Northwestern Hospital specializing in breast cancer. Her areas of research include the evolution of tamoxifen therapy in breast cancer. Dr. O'Regan is studying novel selective estrogen receptor modulators-SERM, an area in which she has studied under one of the world's leading authorities, Dr. V. Craig Jordan at Northwestern. She has received numerous awards including the compassionate care award from the Women's Board of Northwestern Hospital and has several seminal publications on mechanisms of resistance of SERM's among her more than 30 peer-reviewed publications. Dr O'Regan is an Assistant Professor in the Department of Hematology and Oncology in the Winship Cancer Institute at Emory University. She is not under the supervision of the PI and Co-PIs. She doesn't have a conflict of interest for this study. Dr. Ruth O'Regan's biosketch is enclosed.

Appendix C

Case Report Form

Study Title: Early Detection of Breast Cancer Using Molecular Beacon

PI: Dr. Lily Yang,

Co-PI: Dr. William C Wood, Grant W. Carlson and Melinda M. Lewis

Study ID number			
Enrollment groups	**Breast cancer patients**		
Age:	**Race:**		
Physician's name			
Consent date	**Obtained by:**		
FNA date	**Performed by:**		
Ductal lavage date	**Performed by**		
Adverse effects during the procedure			
Post procedure care			
Reported adverse effects after the procedure			
Number of slides obtained			
Results of MB-detection	**Survivin**	**Cyclin D1**	**Her-2/neu**
Total number of positive cells			
Number of positive cells in each level of fluorescence intensity	++++ ( ) +++ ( ) ++ ( ) + ( )	++++ ( ) +++ ( ) ++ ( ) + ( )	++++ ( ) +++ ( ) ++ ( ) + ( )
Number of cells expressing more than one marker genes Survivin + Cyclin D1 Survivin + Her-2/neu Cyclin D1 + Her-2/neu Survivin + Her-2+ cyclin D1			
Frozen breast cancer and normal tissues collected: yes ( ), No ( )	Immunostaining results for survivin, cyclin D1 and Her-2/neu expression in frozen tissue sections. Survivin ( ), cyclin D1 ( ), Her-2/neu ( )		
Pathological diagnosis • Cytopathology from ductal lavage • Cytopathology from FNA • Pathology evaluation of breast cancer tissues			
Treatments received after surgery			

Follow up information ***Breast cancer patients*** Recurrence Prognosis Contact by PI or Co-PIs by telephone Yes ( ), No ( ) If yes, any information.	

BIOGRAPHICAL SKETCH

Give the following information for all ***new*** key personnel.
Copy this page for each person.

NAME	POSITION TITLE
Ruth M. O'Regan	Assistant Professor in Hematology/Oncology

EDUCATION/TRAINING *(Beginning with baccalaureate or other initial professional education, such as nursing, and*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University College, Dublin, Ireland	MB, BcH, BAO (MD)	1982-1988	Medicine
Royal College Physicians, Ireland	MRCPI	1992	Medicine
University College, Dublin	Medical Doctorate	2000	Oncology

A. Professional Experience

7/03-present	**Assistant Professor**	**Emory University, Atlanta, GA**
9/02-6/03	**Assistant Professor**	**Northwestern University, Chicago, IL**
7/99-8/02	**Clinical Instructor**	**Northwestern University, Chicago, IL**
7/98-6/99	**Resident (Internal Medicine PGY3)**	**Northwestern University, Chicago, IL**
7/95-6/98	**Fellow (Hematology/Oncology)**	**Northwestern University, Chicago, IL**
7/94-6/95	**Resident (Internal Medicine PGY3)**	**Medical College Wisconsin, Milwaukee, WI**
7/92-6/94	**Fellow (Oncology)**	**Mater Hospital, Dublin, Ireland**
7/91-6/92	**Resident (Internal Medicine)**	**Meath Hospital, Dublin, Ireland**
7/88-6/91	**Resident (Internal Medicine)**	**Mater Hospital, Dublin, Ireland**

Honors, Awards, and Membership

Compassionate Care Award , Womens' Board of Northwestern Hospital 1997
Coakley Medal from UCD North American Alumni 1999
NSABP Young Clinical Investigator Award 2001
American Society of Clinical Oncology
American Society of Cancer Research
University College Dublin Medical Graduates Association of North America

B. Selected Peer Reviewed Publications

1. **Shah AP, Parmar S,** O'Regan R. **Right atrial and ventricular thrombus infiltrated with osteoblastic osteosarcoma. J Cardiovasc Pharmacol Ther 8;307-311, 2003**
2. **Dardes RC,** O'Regan RM, **Gajdos C, Robinson SP, Bentrem D De Los Reyes A, Jordan VC. Effects of a new clinically relevant antiestrogen (GW5638) related to tamoxifen on breast and endometrial cancer growth in vivo. Clin Cancer Res 6;1995-2001, 2002**
3. O'Regan RM, **Gajdos C, Dardes RC, De los Reyes A, Park WC, Jordan VC. Effects of raloxifene after tamoxifen on breast and endometrial cancer growth.** J Natl Cancer Inst **20;274-83, 2002**
4. **Dardes RC, Bentrem D,** O'Regan RM, **MacGregor-Schafer J, Jordan VC. Effects of the new selective estrogen receptor modulator LY353381.HCL (Arzoxifene) on human endometrial cancer growth in athymic mice.** Clin Cancer Res **7;4149-4155, 2001**
5. MacGregor-Schafer J, Lee ES, Dardes R, **O'Regan RM**, Jordan VC. Analysis of cross-resistance of the selective estrogen receptor modulators arzoxifene (LY353381) and LY 117018 in tamoxifen-stimulated breast cancer xenografts. **Clin Cancer Res** 7;2505-12, 2001
6. **Lee, ES, MacGregor-Schafer, J, Yao, K, England G,** O'Regan RM, **De Los Reyes, A, Jordan VC. Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice.** Clin Cancer Res **6:4893-4899, 2000**

7. **MacGregor-Schafer JI, Lee ES,** O'Regan RM, **Jordan VC. Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice.** Clin Cancer Res **6:4373-4380, 2000**
8. **Yao K, Lee ES, Bentrem D, England G, MacGregor-Schafer JI,** O'Regan RM, **Jordan VC. Antitumor action of physiologic estradiol on tamoxifen-stimulated tumors in athymic mice.** Clin Cancer **Res 6:2028-2036, 2000**
9. O'Regan RM, **Cisneros A, England GM, MacGregor JI, Muenzner HD, Assikis V, Bilimoria MM, Piette M, Dragan Y, Pitot HC, Chatterton R, Jordan VC. Growth characteristics of human endometrial cancer transplanted in athymic mice and treated with new antiestrogens, toremifene and ICI 182,780.** J Natl Cancer Inst 90:1552-1558, 1998.
10. Tonetti, DA, **O'Regan RM**, Tanjore S., England G., and Jordan, VC. Antiestrogen-stimulated human endometrial cancer growth; Laboratory and clinical considerations. **J. Ster. Biochem. Mol. Biol. 65:181-189, 1998**.
11. Eustace S, **O'Regan R,** Graham D, Carney D. Primary multifocal Hodgkin's disease confined to the bone. **Skeletal Radiology 24:61-63, 1995**
12. Kaklamani V, **O'Regan RM.** New targeted therapies in breast cancer. Sems in Onc (in press)
13. Gradishar WJ, **O'Regan RM**. Progress in systemic adjuvant therapy of early stage breast cancer. Int J Clin Oncol 8;239-247, 2003
14. Kaklamani VG, **O'Regan RM.** Breast Cancer Prevention: the risks and benefits of drug therapy. Am J Cancer 1:173-178, 2002
15. **O'Regan RM**, Khuri FR. Farnesyl Transferase Inhibitors: The Next Targeted Therapies for Breast Cancer? Endocrine Related Cancer (accepted)
16. **O'Regan RM,** Jordan VC. The evolution of tamoxifen therapy in breast cancer: selective estrogen receptor modulators and down-regulators. **Lancet Oncology** 3;207-14, 2002
17. O'Regan RM, **Jordan VC. Tamoxifen to raloxifene and beyond** Sems in Oncol **28;260-273, 2001**
18. O'Regan RM, **Jordan VC, Gradishar WJ. Tamoxifen and contralateral breast cancer**. J Amer College Surg 188:678-683, 1999
19. **Bentrem DJ,** O'Regan RM, **Jordan VC. New strategies for the treatment of breast cancer.** Breast Cancer **8;265-74, 2001**
20. O'Regan RM, **Gradishar WJ. Selective Estrogen Receptor Modulators in the year 2000.** Oncology **15;1177-85, 2001**
21. O'Regan RM, **Jordan VC. Tamoxifen, a selective estrogen receptor modulator: A prelude to breast cancer prevention**. Int Med **1999**
22. **O'Regan RM**, England GM, MacGregor JI, Yao KA, Muenzner HD, Takei H, Jordan VC. Laboratory models of breast and endometrial cancer to develop strategies for antiestrogen therapy. **Breast cancer 5:211-217, 1998.**
23. Steroid Hormone Receptors. **O'Regan RM**, Badve S, Gradishar WJ. The Breast: Comprehensive Management of Benign and Malignant Diseases (3rd Edition), Bland KI, Copeland EM III (eds.). W.B. Saunders, Philadelphia, PA, in press, 2003.
24. SERMs Other Than Tamoxifen for Chemoprevention. **O'Regan RM,** Gradishar WJ. In Managing Breast Cancer Risk, (eds.). BC Decker, Inc., Ontario, Canada, in press, 2003.
25. **O'Regan, RM,** Jordan VC. Antiestrogens and related drugs; Introduction. In Ratain, Tempero, Skosey. **Outline of Oncology Therapeutics**, 2001
26. **O'Regan RM,** Jordan VC. Chemoprevention of breast cancer. In Bergan R (ed.) **Cancer Chemoprevention** pp137-154, 2001
27. **O'Regan RM.** Chemoprevention of breast cancer. In Gradishar and Wood (eds.): **Advances in Breast Cancer Management** pp 183-207, 2000
28. **O'Regan RM**, Jordan VC. The advantages and disadvantages of tamoxifen. In Khayat and Hortobagyi (eds.): **Progress in Cancer Therapy, Vol II , 1988.**
29. Gajdos C, **O'Regan RM,** Bentrem DJ, Pappas S, Badve S, MacGregor Schafer J, De Los Reyes A, Jordan VC. Paradoxical effect of ICI 182,780 after long-term tamoxifen treatment on breast cancer growth. Proc AACR 43;950:Abs 4705, 2002
30. **O'Regan RM**, Lee E, Dardes RC, Gajdos C, De Los Reyes A, Badve S, Jordan VC. Characteristics of raloxifene-stimulated breast tumors in vivo. Breast Cancer Res Treat 69;287: Abs 443, 2001
31. Dardes RC, **O'Regan RM** and Jordan VC Low dose tamoxifen effectively controls endometrial cancer growth not previously exposed to the drug. Proc AACR 42;240:Abs:1291, 2001

32. Dardes Rc, Bentrem DJ, **O'Regan RM**, MacGregor-Schafer J, Jordan VC. Effects of the antiestrogens Tamoxifen and LY 353,381.HCL (Arzoxifene) on Endometrial Cancer Growth. **Breast Cancer Res Treat** 64;73 (Abs 269), 2000
33. O'Regan RM, **Gajdos C, Dardes R, Bentrem DJ, De los Reyes A, Jordan VC. Effects of raloxifene after tamoxifen on breast and endometrial cancer growth. Proc ASCO 20;25a:Abs 95, 2001 (oral presentation)**
34. MacGregor-Schafer JI, Lee ES, Yao K, **O'Regan RM**, Jordan VC. Cross resistance to idoxifene and the raloxifene analoque LY 117,018 in the novel tamoxifen stimulated T47D xenograft model. **AACR**, San Francisco 2000
35. **O'Regan RM,** Tonetti DA, Jordan VC (1998) Comparison of tamoxifen and raloxifene on the growth of endometrial cancer cell lines. **Breast Cancer Res Treat Abs 441:306, 1998**
36. **O'Regan RM,** Cisneros A, Jordan VC. Is there life after tamoxifen? New Anti-oestrogens. **Eur J Cancer** 34:S57, 1998
37. **O'Regan RM**, England GM, Cisneros A, Jordan VC. Relationship of tamoxifen dose and the growth of tamoxifen-stimulated tumors. **Proc ASCO Abs 419, 1998.**
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C. Research Support

Ongoing Research Support

None

Completed Research Support

Avon Cosmetics Foundation	2000-2001
Avon Cosmetics Foundation	2001-2002

EMORY HEALTHCARE
EMORY HOSPITALS

Consent to Surgical or Medical Treatment

Date: ___/___/___ Time: _____ Room Number _____

Diagnosis: _____ Procedure: _____

1. I understand the following about the procedure described above:
   a. **Nature and purpose of procedure** (Describe in laymen's terms): _____
   b. **Material risks of procedure:** DEATH, RESPIRATORY ARREST, CARDIAC ARREST, BRAIN DAMAGE, DISFIGURING SCAR, PARAPLEGIA OR QUADRIPLEGIA, PARALYSIS OR PARTIAL PARALYSIS, LOSS OR FUNCTION OF ANY LIMB OR ORGAN, SEVERE LOSS OF BLOOD, ALLERGIC REACTION AND INFECTION. These are material risks of any surgical procedure.
   Other risks of this procedure are: _____
   c. **Likelihood of success:** ☐ Good ☐ Fair ☐ Poor
   ☐ Unknown because: _____
   d. **Practical alternatives to procedure:** ☐ None
   ☐ Other: _____
   e. **Prognosis if procedure rejected:** ☐ Good ☐ Fair ☐ Poor
   ☐ Unknown because: _____
   f. ☐ **If applicable, DNR Order or DNI/Special Code status suspended unless indicated otherwise:**

2. **Consent:** The procedure identified above has been explained to me and all of my questions have been answered. I acknowledge that no guarantees have been made concerning the outcome of the surgical or medical treatment, and I realize that the practice of medicine and surgery are not an exact science. I hereby consent to the procedure by Dr. _____ and/or any assistants who may be present. I also consent to the administration of anesthesia to be applied by or under the direction and supervision of the Section of Anesthesiology of The Emory Clinic.

3. I realize that, during the procedure, the physician/surgeon may become aware of conditions which were not apparent before the start of the procedure, or may determine that additional or different operations or procedures are necessary or appropriate. I therefore authorize and request that the above named physician/surgeon and/or any assistants who may be present to perform additional or different operations or procedures the physician/surgeon deems necessary or advisable; so long as these additional procedures do not conflict with my stated DNR or DNI/Special Code preferences as indicated above.

4. Any tissue, organ, specimen, member or implant, removed or severed in any operation or procedure, may be retained, preserved, used for scientific or teaching purposes, or disposed of by the Hospital or by the Section of Pathology of The Emory Clinic at the discretion of the Hospital or Section, except for the following:

5. If acceptable to the physician/surgeon, I authorize observers to be present during the surgery or procedure. (☐ Yes ☐ No). I further authorize the physician/surgeon, or his designee, to photograph/videotape me before, during and/or after my surgery or procedure, for the purpose related to my care and treatment and/or for the purpose of medical education (☐ Yes ☐ No).

Witness _____ Patient Signature _____

Signature of person obtaining consent _____ Signature of person authorized to consent for patient _____

☐ Check if telephone consent given Relationship to patient _____

12099 **Over for Blood Transfusion Consent** M Consent 009 1003

For Medical Device Recipients Only

Under Federal law, a hospital must report patient information to a company which manufactures medical devices, under some circumstances, including product recalls. However, the hospital can report your social security number to a manufacturer only with your permission. Your social security number may help the manufacturer identify you in the rare case of a product recall. I ☐ do ☐ do not authorize the hospital to report my social security number to the manufacturer of the medical device I receive.

Additional Authorizations for Surgical or Medical Treatment

The purpose of this section is to authorize repeat identical operations/procedures which have already been explained on the reverse side. If there is any change in the operation or other procedure to be done or in the associated risks, another "Consent to Surgical or Medical Treatment" form is to be completed.

Date	Surgical or Medical Treatment	Patient/Person authorized to consent for patient

Consent for Administration of Blood or Blood Products

Date: ___/___/___ Time: _____

The use of blood and blood products to treat my condition has been explained to me and I have been given an opportunity to ask questions.

I understand those precautions and procedures in selecting donors and in collecting, processing, preserving and administering blood and blood products developed by the American Red Cross, American Association of Blood Banks or the Food and Drug Administration will be followed. This will include testing for hepatitis B surface antigen, hepatitis C, HTLV I and II, and antibodies to HIV (AIDS virus). I further understand that in an emergency situation it may be necessary to administer blood or blood products before all tests have been completed.

I realize that despite all precautions and procedures referred to above, adverse reactions may occur. These reactions include, but are not limited to: fever, chills, allergic reactions, shock and transmission of infection (including, among others, hepatitis infection and AIDS virus infection).

I understand there are no artificial or natural substances which can perform all functions of blood. Failure to transfuse blood or blood products when needed could cause additional medical problems or complicate existing ones. These medical problems or complications could cause serious illness or death.

Most of the time, use of blood from random donors is necessary, but in some cases it may be possible to use one of the following: directed donation (blood from donors I select), autologous donation (blood collected from me before or during surgery), fluid replacement (administration of non-blood volume maintenance fluids). These alternatives have been discussed with me and I hereby consent to receive transfusions of blood or blood products from random donors as deemed advisable by any physician involved in the management of my condition or any complications that may occur.

Witness

Patient Signature

Signature of person obtaining consent

Signature of person authorized to consent for patient

Relationship to patient

☐ Check if telephone consent given

References:

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EMORY UNIVERSITY AFFILIATED HOSPITALS

ONCOLOGY RESEARCH COOPERATIVE
INFORMED CONSENT FOR CLINICAL RESEARCH

STUDY TITLE:

Early Detection of Breast Cancer Using Molecular Beacons

Subtitle: Examination of the Sensitivity of Using Molecular Beacons Targeting Survivin, Cyclin D1 and Her-2/neu mRNAs to Detect Breast Cancer Cells in Ductal Lavage and Aspirates of Fine Needle Biopsy in Breast Cancer Patients

PRINCIPAL INVESTIGATOR: LILY YANG, MD/PHD
CO-INVESTIGATORS: WILLIAM C. WOOD, MD., GRANT W.CARLSON, MD., MELINDA M. LEWIS, MD

You are invited to participate in a research study conducted at Emory University by the above investigators. Your participation in this study is voluntary. You should read the information below, and ask questions about anything you do not understand, before deciding whether or not to participate.

PURPOSE OF THE STUDY

Breast cancer is the most common type of cancer and is one of the leading causes of death among women. However, if breast cancer is detected early, the chances of survival are much better. Mammograms (a special kind of x-ray of the breast) can detect breast cancer early. However, about 20% of women who have mammograms and will not have their early breast cancer detected. Of all patients who have abnormal mammograms, only 10 to 20% are confirmed to be breast cancer by biopsy. Therefore, it is important to develop new ways to diagnose breast cancer at its early stage.

Recently, a new technique has been developed to detect breast cancer cells. This technique uses very small, circular "probes" (called molecular beacons) that specifically look for tumor cells. When these probes are in the cells, they cause a fluorescent signal in breast cancer cells. Thus, cancer cells become "labeled" with bright fluorescent colors that can be distinguished from normal cells. We can identify these labeled cells by looking with a special microscope.

WHAT IS INVOLVED IN THE STUDY?

We are examining the possibility of detecting breast cancer cells using special techniques called ductal lavage or fine needle aspiration.

Ductal lavage is a method to collect cells of breast milk ducts. This procedure involves three steps. First, an anesthetic numbing cream is applied to the nipple area and gentle suction is used to locate the openings of the milk duct on the nipple. Then, a hair-thin catheter is inserted into a milk duct opening and two teaspoons of saline are injected slowly into the duct. Next, the ductal cell fluid is withdrawn through the catheter and collected for cellular analysis with the molecular beacon probes.

Fine needle aspiration is a routine biopsy procedure performed in the outpatient clinic that uses a thin needle on a syringe to draw fluid and/or cellular material from breast tissues. A long, thin needle is inserted through the breast into the area of breast abnormality. The cells collected from this procedure will be sent to research lab.

About Fifty (50) of women with breast cancers will be enrolled in this study to determine the feasibility of detecting breast cancer cells in ductal lavage and fine needle aspiration samples using the molecular beacon technology.

You are invited to take part in this study. Please take your time to make your decision.

During a scheduled fine needle aspiration diagnostic procedure, excess aspirates that are not needed for diagnosis, and otherwise would be discarded, will be saved and used for research. No extra tissue will be removed for this research.

Ductal lavage will be performed during scheduled surgery for removing the breast cancer. In the operating room and after administration of general anesthesia, ductal lavage is performed as described above. This procedure will extend your anesthesia time for about 10 minutes.

After removing the breast cancer, if there is excess breast cancer tissue that is not needed for pathological diagnosis, it will be collected and frozen in liquid nitrogen for the examination of the expression of tumor marker genes.

Yes, I am willing to have above procedures performed and specimen donated.
Please initial _____.

HOW LONG WILL I BE IN THE STUDY?

For you, the study will last as long as your scheduled examination, diagnostic and surgical procedures for the treatment of your diseases. Your medical records may be reviewed during the study to determine the sensitivity and specificity of the detection method. Additionally, your prognosis will be followed for three years by examining your medical records and phone calls from the PI or CO-PIs.

WHAT ARE THE RISKS OF THE STUDY?

A recent study on over 500 high risk women conducted at 19 breast cancer centers showed that ductal lavage is a well-tolerated procedure. The majority patients said that the procedure was no more uncomfortable than a mammogram and some felt the sensation as breast fullness. There was no serious side effect found in this study.

Ductal lavage will be collected during the surgery and fine needle aspirates will be obtained from scheduled biopsy. Participation in the study will not add to any discomforts or significant risk to the patients. It is possible that prolongation of general anesthesia for 10 minutes during ductal lavage may have a minimal healthy risk. However, you will be carefully monitored by surgical and anesthetic staff during the ductal lavage procdure to reduce any healthy risk.

WHAT THE PRECAUTIONS TO BE OBERVED BY THE PARTICIPATE BEFORE AND AFTER STUDY PROCEDURES

Please inform your physician if you have fever or feel pain in your breast before or after the fine needle biopsy. Please also contact your physician if you feel swelling in you breast or notice any discharges from your breast nipple after the procedure.
Your ductal lavage procedure will be performed before your surgery to remove the tumor. Your physician will inform you about all precautions for preparation of the surgery. All routine post operation care procedures will be followed and your physician will follow-up on your recovery after the surgery.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

You should not expect your condition to improve as a result of participating in this research. However, your participation in this study may help us learn more about the diagnosis of breast cancers and help doctors better identify and care for breast cancer in the future.

WHAT OTHER OPTIONS ARE THERE?

You have the right to refuse to participate in this study. Choosing not to take part will not result in any penalty or loss of benefits to which you are entitled.

Your participation may be terminated by your physician or principal investigator if you are no longer meet the inclusive criteria for the study, such as becoming pregnant or starting chemotherapy before the procedures are performed.

WHAT ABOUT CONFIDENTIALITY?

All participants of this study will be assigned a Study ID number. We will use the study number rather than your name on study records where we can. Your name and other facts that might point to you will not appear when we present this study or publish its results.
People other than those doing the study may look at both medical charts and study records. Agencies that make rules and policy about how research is done have the right to review these records. The Department of Defense has the Congressionally Directed Medical Research Programs (CDMRP) funding research on the diagnosis and treatment of breast cancer. This study is sponsored by this program. Those with the right to look at your study records are, the Department of Defense, the National Cancer Institute, the Emory Clinic, Emory University Hospital and the Emory University Institutional Review Board. Records can also be opened by court order. We will

keep your records private to the extent allowed by law. We will do this even if outside review occurs.

WHAT ARE THE COSTS?

There is no added cost to you for taking part in this study.

Other than medical care that may be provided specially stated in the consent form, there is no other compensation available for your participation in this research.

If you are hurt or get sick because of this research study, you need to notify your physician immediately. The PI and CO-PIs will make appropriate arrangement for you to receive medical care at an Army hospital or clinic free of charge. You will only be treated for injuries that are directly caused by the research study. The Army will not pay for your transportation to and from the hospital or clinic.

If you have questions about this medical care, talk to the principal investigator for this study, (Lily Yang, 404-778-4269). If you pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact the principal investigator. If the issue cannot be resolved, contact the U. S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at (301) 619-7663/2221.

Emory University has not set aside funds to pay for your care or to compensate you if you are injured.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled.

This is a feasibility study for the detection of breast cancer cells using molecular beacon. Since these results have not been validated and accepted as reportable results, the test results will not be provided to you.

During this study you will be asked to provide fine needle biopsy, ductal lavage and breast cancer tissue samples. There samples will be used for detection of breast cancer cells using molecular beacon and examination of tumor marker gene expression in breast cancer cells. They may be used for purpose that currently unknown. There is a chance that the samples that you are donating under this study may be used in other research studies and may have some commercial value. Should your donated samples lead to the development of a commercial product, Emory University will own it and may take action to patent and license the product. Emory University does not intend to provide you with any compensation for your participation in this study nor for any future value that the sample you have given may be found to have. You will not receive any notice of future uses of your samples.

WHAT IF NEW INFORMATION ABOUT THIS TREATMENT IS LEARNED?

We may learn new things during the study that you may need to know. If so, you will be notified about any new information.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, contact your physician or Dr. Lily Yang, Principal Investigator, at 404-778-4269.

For questions about your rights as a research participant, contact Dr. James W. Keller, Chairman of the Emory University Institutional Review Board (IRB) at (404) 727-5646. The IRB is a group of people who review the research to protect your rights.

WHO CAN BE INCLUDED IN THIS STUDY?

Patients of all ages and races diagnosed with breast cancers or with possibility of breast cancer, who come to Emory Clinic or Emory Hospital.

WHO SHOULD BE EXCLUSED FROM THIS STUDY?

Breast cancer patients who have received chemotherapy or radiation therapy within last three months.

Breast cancer patients who are currently pregnant or nursing.

Brest cancer patients who can not speak and read English.

CONSENT

If you agree to participate in this study, please sign below.
You are entitled to have a copy of the consent, regardless if you sign the document.

Please type your name_____

Signature _____ _____ _____
Participant Date Time

Or

Please type your name_____

Signature _____ _____ _____
Legally authorized representative Date Time

Permanent Address of the Participate

Street_____, Apt_____

City_____, State_____, Zip_____

Please type your name_____

_____ _____ _____
Person obtaining consent Date Time

_____ _____ _____
Principal Investigator (if different from above) Date Time

SIGNATURE OF WITNESS:

My signature as witness certifies that the subject signed this consent from in my presence as her voluntary act and deed.

_____
Name of Witness

_____ _____
Signature of Witness Date (Same as subject's)

Date of Approval:

Expiration Date:

WINSHIP CANCER INSTITUTE

INFORMED CONSENT ADDENDUM

FOR

TISSUE STORAGE/FUTURE RESEARCH

As a participant in the study on "Early Detection of Breast Cancer Using Molecular Beacons", I voluntarily donate ductal lavage, fine needle aspirates and breast cancer tissue samples to Emory University. These samples will be used for examination of the presence of tumor markers using molecular beacons, types of tumor marker gene expression in breast cancer cells and studies on early detection of breast cancer. There is a possibility that these samples that I am donating under this study may be used in other research studies and may have some commercial value. Should my donated sample(s) lead to the development of a commercial product, Emory University will own it and it is possible that it will be patented and licensed by Emory University. Emory University does not intend to provide me with any compensation for my participation in this study and will not give me any notice of future uses of my sample(s).

I am informed that my identity will be confidential and a specific ID number will be used to identify my tissue. The link between that number and my name will be carefully guarded. My tissue samples will be used only for research in Dr. Lily Yang's lab, and will not be sold. If I decide now that my tissue can be kept for research, I can change your mind at any time. Just contact Dr. Lily Yang at 404-778-4269 or Lyang02@emory.edu, and let her know that I do not want her to use my tissue. She can link my name to my specimen. My donated samples can be destroyed at anytime. I may agree to participate in the research protocol, but refuse to provide the additional samples discussed above.

Am I willing to donate my left over tissue for possible future research of early detection of breast cancer?

Yes_____ No_____

After making your choice, please sign below.

Participant	Date	Time	
Person obtaining consent	Date	Time	
Principal Investigator (if different from above)	Date		

EMORY UNIVERSITY AFFILIATED HOSPITALS

WINSHIP CANCER INSTITUTE

AUTHORIZATION TO USE AND DISCLOSE PERSONAL HEALTH INFORMATION

TITLE:

- **Early Detection of Breast Cancer Using Molecular Beacons**

PRINCIPAL INVESTIGATOR:

- Lilly Yang, MD/PhD

COINVESTIGATORS:

- William C. Wood, MD
- Grant Carlson, MD
- Toncred Styblo, MD
- Melinda M. Lewis, MD

SPONSOR:

- Department of Defense

INTRODUCTION/PURPOSE:

The United States government has issued a new privacy rule to protect the privacy rights of patients. This rule was issued under a law called the Health Insurance Portability and Accountability Act of 1996 (HIPAA). The Privacy Rule is designed to protect the confidentiality of your health information. This document, called an "Authorization," describes your rights and explains how your health information will be used and disclosed for this study, named above.

This study is being conducted by the Emory University Winship Cancer Institute. The purpose of this research study is to learn if molecular beacons can help with the early detection of breast cancer.

ABOUT CONFIDENTIALITY AND PROTECTED HEALTH INFORMATION (PHI):

Protected health information (PHI) is any health information about you that identifies you or that can reasonably be used to identify you by the person to whom it is provided. The people

who are conducting the study (the "Researchers") may need to look at your medical and study records that contain PHI. In addition, government agencies that make rules and policies about how research is done, including the Office for Human Research Protections (OHRP) [and the Food and Drug Administration (FDA)] and, have the right to review these records. Sponsors who pay for the study also have the right to review records, as does the Emory University Institutional Review Board (IRB) and IRBs at other sites if the study is being conducted at more than one location. In addition, records may be disclosed pursuant to court order.

We will not use or disclose your records in any ways other than the ways we describe in this form, and we will keep your records private to the extent allowed by law. We will do this even if outside review of your records occurs. We will use a study number or other code rather than your name on study records where we can. Your name and other facts that might point to you will not appear when we present this study or publish its results.

Under the Health Insurance Portability and Accountability Act (HIPAA), a federal law enacted to protect the privacy of your PHI, before we can use or disclose your PHI, we must provide you with information about what PHI will be used and how it will be used and disclosed. This section of this form provides you with this information regarding your PHI. Specifically, it will tell you what PHI the Researchers will look at; who will collect the PHI; who will use the PHI, with whom it will be shared and the purpose of each use or disclosure; the expiration date or event, if any, after which we won't use or disclose your PHI any more; and your rights under HIPAA to ask us not to use your PHI any more. If you decide to participate in this research, then you will be agreeing to let the Researchers and any other persons, companies or agencies described below to use and share your PHI for the study in the ways that are set forth in this section, so please review this section very carefully.

WHAT PHI WILL THE RESEARCH TEAM USE?

The Researchers will look at your entire medical file, which contains all of your personal identifying information and health insurance information; health care providers notes; results of laboratory tests, x-rays and other medical tests; results of physical examinations, and any other information that your health care provider may have recorded about your health or health care. The researchers at the Winship Cancer Institute will also add your PHI to a database that they are compiling for research purposes. You will be followed-up through your doctor's office visits and telephone calls by the Researchers listed above for three years. The results of your laboratory tests, mammograms, response to treatments and prognosis will be added to your data files.

WHO WILL COLLECT THE PHI?

The Researchers will collect and copy the PHI described above during your doctor's office visits and after your operation or treatment from your records or by calling you directly. If any of the PHI is to be shared with other persons, as described later on in this section, then the Researchers also will be responsible for making these disclosures.

WHO WILL USE THE PHI; WITH WHOM WILL IT BE SHARED; AND FOR WHAT PURPOSE(S) WILL IT BE USED OR SHARED?

In order to conduct the study, the PHI that is collected regarding you will be used by or shared with the following persons, agencies or companies for the purposes listed in the chart below.

Person/Entity	*Purpose*
Researchers	*To conduct the study entitled, "Early Detection of Breast Cancer Using Molecular Beacons", the purpose of which is to learn if molecular beacons can help with the early detection of breast cancer.*
Governmental Agencies with oversight over the research being conducted, including the FDA and OHRP	*To monitor safety, efficacy and compliance with applicable laws and regulations.*
University personnel, committees and departments charged with oversight of research, including the IRB.	*To monitor safety and compliance with applicable laws, regulations and University policies and procedures.*
The US Department of Defense, the study sponsor.	*To provide oversight for the study and to perform data analysis.*
Study monitors hired by the Department of Defense	*To verify that data has been properly collected for reporting to the FDA.*

EXPIRATION DATE OR EVENT:

The Researchers will add your PHI to a database that they are compiling for research purposes. There is no date or event after which your Authorization will expire and your PHI will no longer be used for this purpose. After the study is finished and the results are published, any records connecting your personal information to results of laboratory tests, x-rays, other medical tests and physical examinations will be erased from the our database or hard copies will be destroyed.

YOUR RIGHT UNDER HIPAA TO REVOKE YOUR AUTHORIZATION AND ASK US NOT TO USE YOUR PHI ANY MORE:

Giving the Researchers your authorization to use and share your PHI is voluntary. At any time, you may choose to revoke your authorization for the Researchers to use and share your PHI. If you revoke your authorization, the Researchers may no longer be able to provide you with any research-related treatment, but your revocation will not otherwise affect your current or future health care. Further, if you revoke your authorization, there will be no penalty or loss of any benefits to which you are otherwise entitled.

If you decide that you want to revoke your authorization for us to use your PHI, you may do so by providing it to the researcher a written and signed request to do so. Once we receive your written revocation of your authorization to use your PHI, we will not make any other use of your PHI or share it with anyone else, except as follows: (a) we will let the study sponsor (if any) know that you have revoked your authorization; (b) we will not ask the study sponsor (if any) or any other parties to whom we said we would disclose data to return any data that we provided to it/them before you revoked your authorization; (c) and, even after we receive your revocation, we will still provide the study sponsor (if any) and any other parties to whom we stated that we would disclose data with any data that is necessary to preserve the integrity of the research study, and we will provide any governmental or University personnel, departments

or committees with any data that they may need in order to comply with/or investigate adverse events or non-compliance with any applicable laws, regulations or University policies.

PHI MAY BE RE-DISCLOSED:

If we disclose your PHI to one of the other parties described above, that party might further disclose your PHI to another party. If your PHI is further disclosed, then the information is no longer covered by HIPAA.

SIGNATURE AND DATE:

The researchers will ask you to sign and date this form.
You will be provided with a copy of this form after you have signed and dated it.

AUTHORIZATION

I have read this authorization form and have been given the chance to ask questions about it. I am signing this form voluntarily and I understand that by signing I will be authorizing the Researchers to use and disclose my PHI as described in this form.

_____ _____
Signature of: **Date**

____ **Participant or**

____ **Participant's Representative**
[check one]

_____ _____
Witness **Date**

IF APPLICABLE

For Personal Representatives Signing for Participants who are Unable to Sign due to Incapacity:

I certify that I _____, am over 18 years of age and that I am the personal representative of _____ ("Participant"), a person over 18 years of age, who has been invited to participate in this study but who is unable to sign this form due to physical or mental incapacity. I certify that legally I have been designated as the personal representative of the Participant because [insert description of reason for authority, e.g., "I have a court order dated 0/0/0 naming me as the Participant's legal representative"; I am named as the representative by a Durable Power of Attorney for Healthcare dated 0/0/0," etc.]. I further certify that I have full legal authority to make decisions concerning the participant, including decisions regarding health care and health care information.

Real-time Detection of Gene Expression in Cancer Cells Using Molecular Beacon Imaging: New Strategies for Cancer Research

Xiang-Hong Peng,[1] Ze-Hong Cao,[1] Jin-Tang Xia,[3] Grant W. Carlson,[1] Melinda M. Lewis,[2] William C. Wood,[1] and Lily Yang[1]

Departments of [1]Surgery, Winship Cancer Institute and [2]Pathology, Emory University School of Medicine, Atlanta, Georgia and [3]The First People's Hospital of Guang Zhou, Guang Zhou, P.R. China

Abstract

Development of novel approaches for quantitative analysis of gene expression in intact tumor cells should provide new means for cancer detection and for studying the response of cancer cells to biological and therapeutic reagents. We developed procedures for detecting the levels of expression of multiple genes in fixed as well as viable cells using molecular beacon imaging technology. We found that simultaneous delivery of molecular beacons targeting survivin and cyclin D1 mRNAs produced strong fluorescence in breast cancer but not in normal breast cells. Importantly, fluorescence intensity correlated well with the level of gene expression in the cells detected by real-time reverse transcription–PCR or Western blot analysis. We further show that molecular beacons can detect changes of survivin gene expression in viable cancer cells following epidermal growth factor stimulation, docetaxel treatment, and overexpression of *p53* gene. Thus, molecular beacon imaging is a simple and specific method for detecting gene expression in cancer cells. It has great potential for cancer detection and drug development. (Cancer Res 2005; 65(5): 1909-17)

Introduction

Development of new approaches for detecting cancer cells and determining the responses of the cells to therapeutic reagents holds great promise to increase the survival of cancer patients. It is well known that human cancer cells develop due to abnormalities in gene expression that provide growth advantages, metastatic potential, and apoptosis resistance to the cells (1–3). Methods for specific detection of abnormal gene expression in intact single cancer cells should provide new tools for identifying cancer cells in clinical samples, studying biological effects, and evaluating the effects of therapeutic reagents on specific molecular targets in cancer cells.

In this study, we developed a molecular beacon fluorescence imaging approach to detect the levels of expression of multiple genes simultaneously in single cells. Molecular beacons are stem-loop type oligonucleotide probes dual-labeled with a fluorophore and a quencher. In the absence of the target, the stem brings the fluorophore and quencher molecules together, which prevents the production of a fluorescent signal. When the molecular beacon hybrids to its specific target sequence, the stem is forced to break apart, which enables it to generate a fluorescent signal (4–6). Because binding conditions between the loop and complementary target sequences are very stringent, only a target with perfectly matching sequences is able to hybridize to the molecular beacon (5). During the last several years, molecular beacon technology has been used in various applications to detect oligonucleotides in solution, including DNA mutation detection and real-time quantification of PCR products and protein-DNA interaction (6–8).

The ability of molecular beacon probes to detect specific target molecules without separation of unbound probes also provides an opportunity to detect intracellular mRNA molecules in intact cells. The feasibility of detecting intracellular mRNA has been examined in several laboratories (9–13). It has been shown that molecular beacons were able to visualize mRNA molecules in several human and animal cell lines after introducing into cells through microinjection or liposome delivery (9–11, 14). It has also been shown that the detection limit of preformed molecular beacon/β-actin mRNA duplexes microinjected into the cells is 10 mRNA molecules, suggesting that molecular beacon technology is a very sensitive method for detecting mRNAs in cells (9).

Although previous studies suggested that detection of intracellular mRNA using molecular beacons is a feasible approach, the question remains of how to develop this novel technology into a simple procedure that can be used broadly in basic research and clinical laboratories. To address this issue, we developed procedures that enable us to detect gene expression in fixed as well as viable cells. We designed molecular beacons targeting survivin and cyclin D1 mRNAs, which are highly expressed in breast cancer cells (15, 16). Survivin is a member of the inhibitor of apoptosis protein family that plays a crucial role in the apoptosis resistance of tumor cells (17). Increasing evidence indicates that survivin is also a promising tumor marker because it is normally expressed during fetal development but is not expressed in most normal adult tissues (18). However, high levels of survivin are detected in many human cancer types including 70% of breast cancers (16, 19). Also, cyclin D1, an important regulator of cell cycle, is overexpressed in 50% to 80% of breast cancer tissues, whereas it is low or absent in normal breast tissues (15). In this study, we examined the feasibility of detecting expression of survivin and cyclin D1 genes in human breast cancer cells using the molecular beacon–imaging technology.

Materials and Methods

Human Breast Cancer or Normal Cell Lines and Tissues

Breast cancer cell lines SKBr-3, MDA-MB-231, and MCF-7 and normal immortalized human mammary epithelial cell line MCF-10A were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-435 cell line was provided by Dr. Zhen Fan (MD Anderson Cancer Center, Houston, TX).

Requests for reprints: Lily Yang, Department of Surgery and Winship Cancer Institute, Emory University School of Medicine, 1365 C Clifton Road Northeast, Atlanta, GA 30322. Phone: 404-778-4269; Fax: 404-778-5530; E-mail: Lyang02@emory.edu.

Frozen human breast cancer and normal tissues were obtained according to an approved institutional review board protocol at Emory University from breast cancer patients during surgery to remove the tumors. Tissues were frozen immediately in liquid nitrogen and kept at −80°C.

Design and Synthesis of Molecular Beacons

The sequences of molecular beacons targeting survivin or cyclin D1 mRNAs were unique for each gene. These include (*a*) survivin MB-FITC: 5′-FITC-TGGTCCTTGAGAAAGGGC*GACCA*-Dabcyl-3′, (*b*) survivin MB-Cy3: 5′-Cy3-*C*TGAGAAAGGGCTGCCAG*TCTCAG*-Dabcyl-3′, and (*c*) Cyclin D1 MB-Texas Red: 5′-Texas-Red-TGGAGTTGTCGGTGTAGA*CTCCA*-Dabcyl-3′. Control molecular beacons for targeting human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GAPDH MB-Cy 3 or GAPDH MB-6-FAM, were also synthesized as the following: 5′-Cy3 or 6-FAM-*C*GAGTCCTTCCACGATAC-CC*ACTCG*-Dabcyl-3′. The underlined bases were those added to form a stem with an optimal Tm condition. All molecular beacons were synthesized by MWG-Biotech Inc. (High Point, NC).

The specificity of the molecular beacons in solution was determined using synthesized oligonucleotide targets (Sigma Genosys, Woodlands, TX). These include (*a*) survivin target: 5′-CCTGCCTGGCAGCCCTTTCTCAAG-GACCACCGCATCTCTACATTCAAGAAC-3′, (*b*) cyclin D1 target: 5′-AGAAGCTGTGCATCTACACCGACAACTCCATCCGGC-3′, (*c*) HER-2/*neu* gene target: 5′-AGTGTGCACCGGCACAGACATGAAGCTGCGGCTCCCT-3′, and (*d*) K-*ras* gene: 5′-GTAGTTGGAGCTGGTGGCGTAGGCAA-GAGTGCCTTGACGATACAGCTAATT CAG-3′. Survivin or cyclin D1 molecular beacon (200 nmol/L) was mixed with 1 μmol/L of various DNA targets in 100 μL of Opti-MEM (Invitrogen, Carlsbad, CA). After incubating at 37°C for 60 minutes, fluorescence intensity was measured by a fluorescence microplate reader (Bioteck FL600 fluorometer, Winooski, VT).

Real-time Reverse Transcription–PCR

Total RNAs were isolated and amplified with an Omniscript reverse transcription kit (Qiagen Inc, Valencia, CA). Real-time PCR was done on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). The primer pairs for detecting the expression of survivin gene were survivin forward 5′-TCCACTGCCCCACTGAGAAC-3′ and survivin reverse 5′-TGGCTCCCAGCCTTCCA-3′. PCR primers for cyclin D1 were forward 5′-AGAAGCTGTGCATCTACACCGACAACTC-CATCCGGC-3′ and reverse 5′-GGTTCCACTTGAGCTTGTTCACAA-3′. The primer pairs for β-actin gene were β-actin forward, 5′-AAAGACCTGTA CGCCAACACAGTGCTGTCTGG-3′, and β-actin reverse, 5′-CGTCA-TACTCCTGCTTGCTGATCCACATCTGC-3′, and for GAPDH were forward 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse 5′-CATGTGGGC-CATGAGGTCCACCAC-3′.

Western Blot Analyses

Cell lysates were collected after different treatments and total cellular protein was resolved on polyacrylamide SDS gels. Western blot analysis for the level of survivin protein was done according to a standard protocol as described (19). The membranes were incubated for 1 hour with goat anti-human survivin (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal antibodies to β-actin (Sigma Chemical Co, St. Louis, MO). The levels of specific proteins in each lysate were detected by enhanced chemiluminescence using ECL plus (Amersham International, Buckingham, United Kingdom) followed by autoradiography.

Detection of Gene Expression in Fixed Cells

Cells were plated on chamber slides for 24 hours and then fixed with ice-cold acetone for 5 to 10 minutes. The slides were stained with a mixture of 200 nmol/L of survivin MB-FITC and cyclin D1 MB-Texas Red in Opti-MEM at 37°C for 60 minutes and then examined using a confocal microscope (LSM 510 Meta, Carl Zeiss Microimaging, Inc., Thornwood, NY).

For detecting survivin gene expression on tissue sections, 5-μm frozen sections of breast normal and cancer tissues fixed with ice-cold acetone were incubated with 200 nmol/L survivin MB-Cy3 for 60 minutes and then counterstained with 10 μg/mL Hoechst 33342 (Molecular Probes, Inc., Eugene, OR). For immunofluorescence labeling, acetone-fixed frozen sections were incubated with a goat anti-human survivin antibody and then with FITC-conjugated anti-goat antibody. For double-labeling survivin MB and human endothelial cell marker CD31, tissue sections were incubated with survivin MB-Cy3 and then with an anti-CD31 antibody followed by a FITC-conjugated secondary anti-mouse antibody. One tissue section was double-labeled with goat anti-human survivin and mouse anti-human CD31 antibodies followed by FITC-labeled donkey anti-goat antibody or biotinylated horse anti-mouse antibody and Texas Red avidin. The tissue slides were observed under a Nikon fluorescence microscope (Nikon Eclipse E800, Nikon Instruments Inc. Melville, NY). Fluorescence images were taken using an Optronics Magnafire digital imaging system (Meyer Instruments, Houston, TX).

Quantification of the Level of Gene Expression in Viable Cells Using Molecular Beacons

FACScan Analysis. Cells were plated in six-well plates and cultured in medium containing 2% fetal bovine serum overnight. The cells were transfected with 400 nmol/L of either survivin MB-FITC or GAPDH MB-6FAM using LipofectAMINE 2000 in Opti-MEM (Invitrogen). Three hours after transfection, 100 ng of human recombinant epidermal growth factor (EGF; Invitrogen) were added to the EGF-treated group for 1 hour and the cells were collected for FACScan analysis (Becton Dickinson, Mansfield, MA).

Cells transduced with an adenoviral vector expressing a wild-type *p53* gene (Adp53, Qbiogene, Carlsbad, CA) or control adenoviral vector (Adcmv) at a multiplicity of infection of 50 plaque-forming units for 24 hours were collected and divided into two groups. One group of the cells was transfected with 400 nmol/L of survivin MB-FITC and the other was transfected with 400 nmol/L of GAPDH MB-6-FAM using LipofectAMINE 2000 in Opti-MEM (Invitrogen). Fluorescence intensity of the cells from all groups was examined using FACScan analysis.

Fluorescence Microplate Reader. Cells were plated in 96-well culture plates at 80% confluence for 24 hours. EGF-treated and nontreated groups were cultured in the medium with 2% fetal bovine serum. The cells were then transfected with a mixture of 400 nmol/L of survivin MB-FITC and internal control GAPDH MB-Cy 3. At 3 hours after transfection, 100 ng/ml of EGF was then added to the wells in the EGF-treated group and 10 or 50 nmol/L of docetaxel (Aventis Pharma, Bridgewater, NJ) were added to the docetaxel-treated group. The culture plates were immediately placed in the microplate reader and fluorescence units in each well were measured at different time points.

Results

Survivin and Cyclin D1 Molecular Beacons Specifically Bind to DNA Targets. The design of the survivin and cyclin D1 molecular beacons and illustration of the mechanism of binding molecular beacons to specific oligonucleotide targets are shown in Fig. 1*A*. We showed that survivin or cyclin D1 molecular beacon specifically bound to its DNA target and generated 5- to 8-fold higher fluorescent signal when mixed with specific DNA target compared with other targets (Fig. 1*B*).

Detection of Human Breast Cancer Cells Using Molecular Beacons Targeting Tumor Marker mRNAs. We examined whether molecular beacons targeting different tumor marker mRNAs can be labeled with different fluorophores and expression of the tumor marker genes can be determined simultaneously in single cells. We found that a combination of survivin and cyclin D1 molecular beacons detected the expression of both survivin and cyclin D1 genes simultaneously and generated fluorescent signals corresponding to either survivin (green) or cyclin D1 (red) mRNA in the cancer cells (Fig. 2*A*). Importantly, the fluorescent signal was very low for both molecular beacons in a normal immortalized human mammary epithelial cell line (MCF-10A), indicating that survivin and/or cyclin D1 molecular beacons can be used as fluorescence probes for the detection of breast cancer cells (Fig. 2*A*). The results of examination of fluorescence

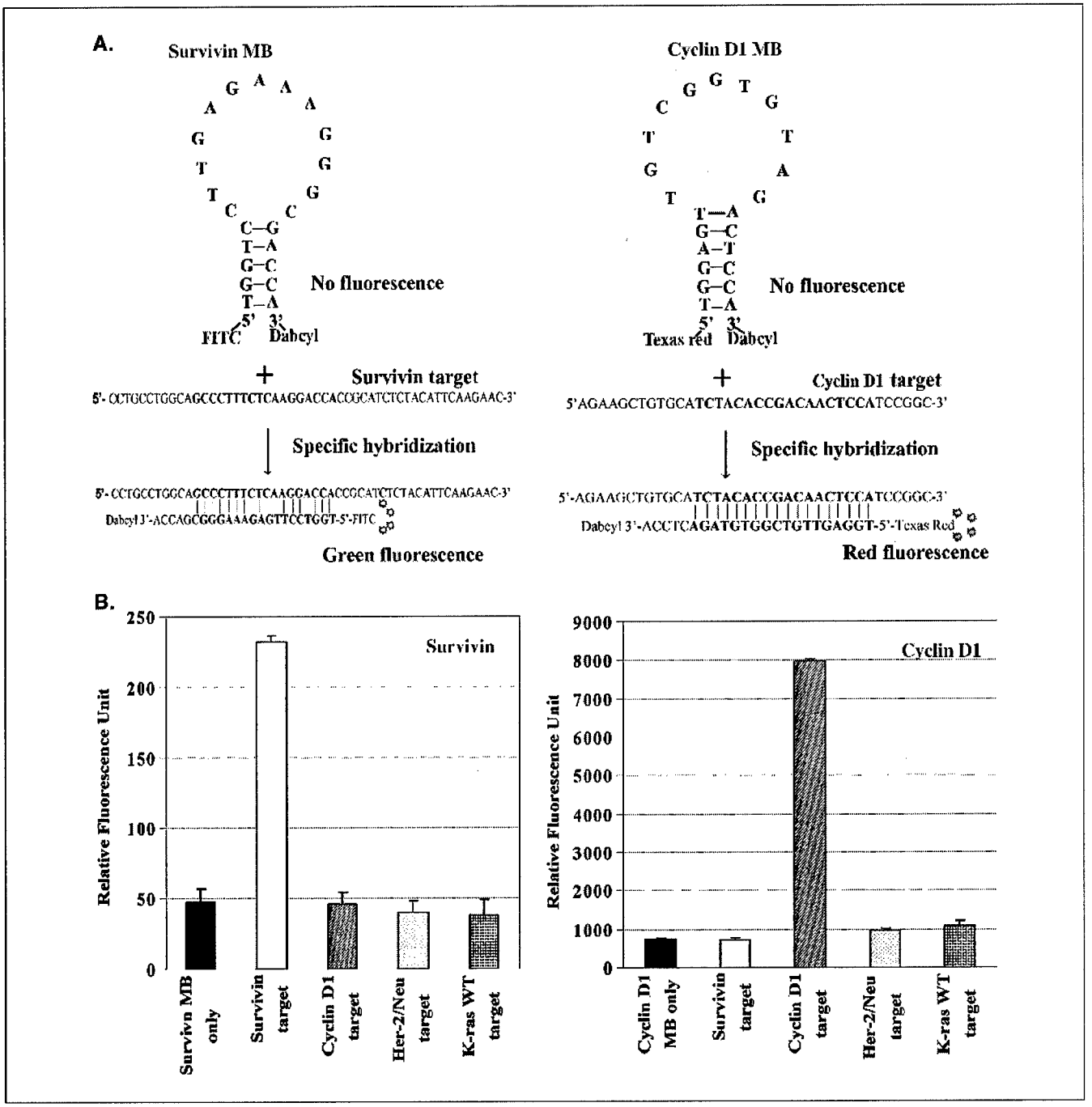

Figure 1. Schematic illustration of molecular beacon design and examination of specific binding of the molecular beacons to their oligonucleotide targets. *A*, both survivin and cyclin D1 molecular beacons have 23 nucleotides with 5′ stem and loop sequences complementary to survivin or cyclin D1 gene. The stem length for survivin molecular beacon is 5 nucleotides with the 5′ end labeled with FITC and the 3′ end labeled with a quencher (*Dabcyl*). Cyclin D1 molecular beacon has a stem containing 6 nucleotides with the 5′ end labeled with Texas Red and the 3′ end with Dabcyl. Survivin and cyclin D1 molecular beacons only generate fluorescent signals when hybridized to their specific DNA target. *B*, examination of specificity of the molecular beacons *in vitro*. Survivin or cyclin D1 molecular beacon was mixed with various synthesized DNA targets. The fluorescence units were measured using a fluorescence microplate reader. Survivin or cyclin D1 molecular beacon only bound and generated strong fluorescent signal when mixed with its specific DNA target. *WT*, wild-type.

intensity and the level of survivin or cyclin D1 gene expression in tumor and normal cell lines further showed that the fluorescent signals detected by the molecular beacons correlated very well with the levels of survivin or cyclin D1 gene expression, both in mRNA and protein levels (Fig. 2*B-D*). For example, MDA-MB-435 and SKBr-3 expressed very high levels of survivin gene, and the strongest fluorescent signal was detected in these cell lines. Conversely, these cell lines expressed low levels of cyclin D1 gene and showed a weak red fluorescence staining (Fig. 2*A-D*). MCF-7 cells expressed a moderate level of survivin gene but had a very high level of cyclin D1 gene expression. Delivery of survivin and cyclin D1 molecular beacons into this cell line produced a strong red fluorescent signal (cyclin D1) and an intermediate level of green fluorescent signal (survivin; Fig. 2*A-D*). Our results show

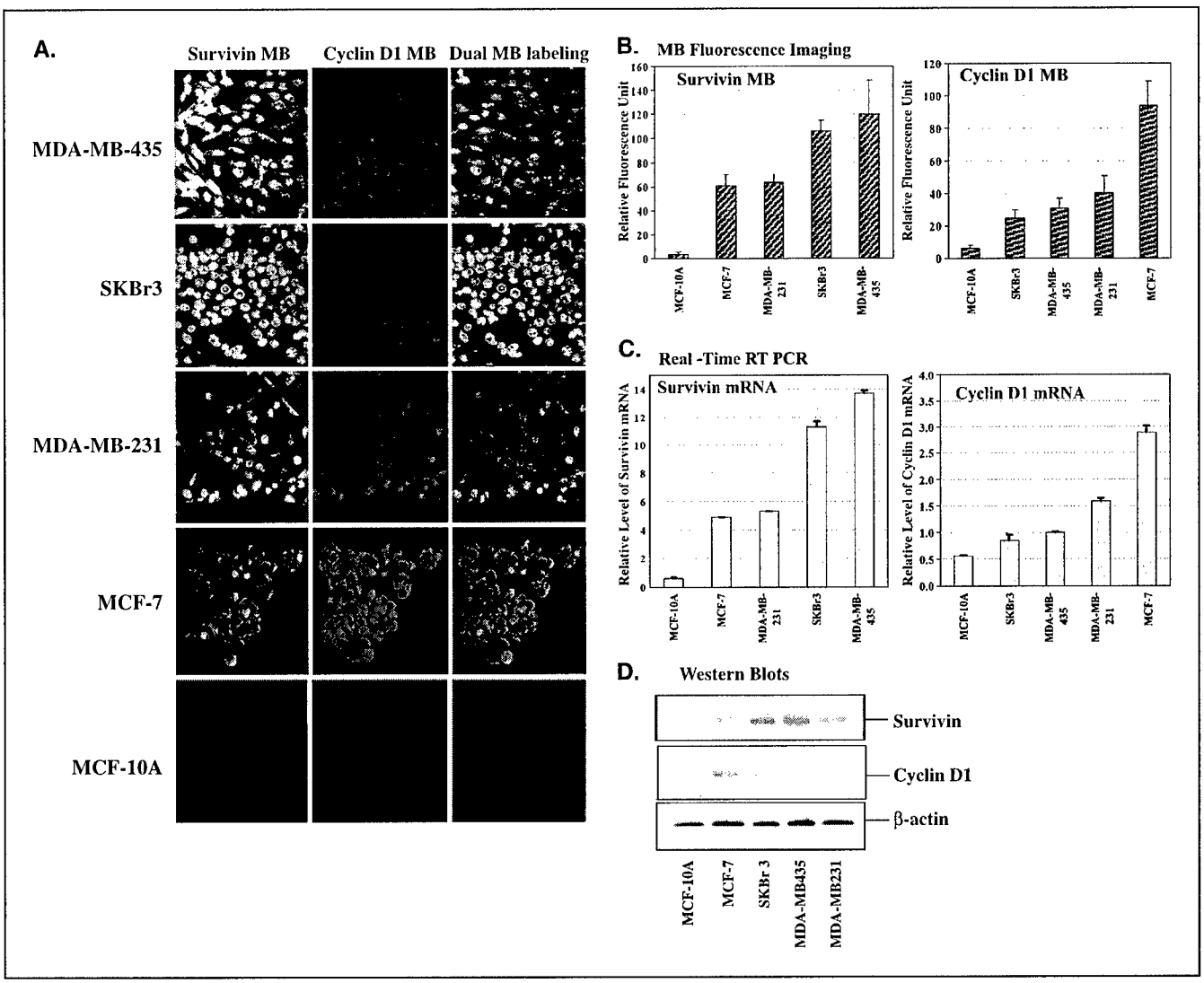

Figure 2. Simultaneous detection of the levels of survivin and cyclin D1 mRNAs in breast cancer cells. *A*, dual molecular beacon (*MB*) imaging of breast cancer cells. A mixture of survivin and cyclin D1 molecular beacons was incubated with the fixed cells and then examined under a confocal microscope. *B*, quantitative analysis of the level of fluorescence intensity produced in breast cancer and normal cells. Fluorescence intensity was determined by measuring the mean fluorescence units from four randomly selected areas for each image taken under a confocal microscope. The mean fluorescence unit from four areas of each cell line is shown in the figure. Similar results were observed in repeat experiments. *C*, detection of the levels of survivin and cyclin D1 mRNAs by real-time RT-PCR. Relative level of survivin or cyclin D1 mRNA was calculated from the quantity of survivin or cyclin D1 PCR products and the quantity of β-actin PCR products. *D*, examination of the levels of survivin protein in tumor and normal cell lines by Western blotting. The levels of survivin or cyclin D1 protein correlated well with the levels of survivin or cyclin D1 mRNA detected *in situ* in fixed tumor cells using molecular beacon detection or with the real-time RT-PCR results.

that a combination of molecular beacon technology with fluorescence imaging is a novel approach to simultaneously detect the levels of multiple gene expressions in intact single cells.

Molecular Beacons Detect Cancer Cells on Frozen Sections of Breast Cancer Tissues. We further developed a simple and fast procedure that allows us to detect survivin gene expression *in situ* on frozen tissue sections. Our previous study showed that survivin is expressed in 72% of breast cancer tissues, including 34 invasive breast ductal carcinoma and 2 lymph node metastases, using Western blot analysis of tissue lysates obtained from frozen tissue samples of the patients with cancer (19). In this study, we examined survivin gene expression on frozen tissue sections of those cancer tissues using survivin MB. We found that survivin MB-Cy3 was able to produce strong red fluorescent signals in breast cancer cells on frozen tissue sections (Fig. 3*A*). A high level of survivin gene expression was consistently detected in the breast cancer cells in nine of nine invasive ductal carcinoma tissues and one lymph node with metastastic lesions that were previously found positive for survivin protein by Western blot analysis. Two breast cancer tissues that were negative for survivin protein expression also lacked survivin MB positive cells (data not shown). Moreover, the survivin MB positive cells were not found in frozen tissue sections of all five paired normal breast tissues (Fig. 3*A* and *B*, representative results of survivin molecular beacon imaging and immunofluorescence labeling with a survivin antibody).

We have also examined the expression of survivin proteins in ductal carcinoma *in situ* (DCIS) tissues by immunohistochemical staining on frozen or paraffin sections using a polyclonal anti-survivin antibody. Eleven of 17 DCIS tissues displayed various levels of survivin protein expression (data not shown). We further

examined frozen tissue sections from two DCIS tissues and found that breast cancer cells in those DCIS tissues were positive for survivin molecular beacon, suggesting that survivin gene expression is an early event in the tumorigenesis of breast cancer (Fig. 3*A*).

In addition, we found that survivin gene–expressing cells in breast cancer tissues included cancer cells as well as cells in the vascular structures (Fig. 3*C*). When the same section was double-labeled with an antibody specific for a CD31 human endothelial cell marker (20), those survivin-expressing cells in the vascular structures were shown to be endothelial cells (Fig. 3*C*). Establishment of this molecular beacon detection method for measuring gene expression *in situ* should provide pathologists with a new tool to identify cancer cells in clinical samples.

Monitoring the Level of Real-time Gene Expression Using Survivin Molecular Beacon. We used three model systems to determine whether survivin molecular beacon was able to detect changes of survivin gene expression in viable cells, including EGF or docetaxel induced up-regulation and tumor suppressor gene *p53*–induced down-regulation of survivin gene expression (21, 22). Breast cancer cells were transfected with a mixture of survivin and GAPDH molecular beacons and observed under a fluorescence microscope after treatment with EGF for 1 hour or docetaxel for 24 hours. Our results showed that treatment of the cells with EGF or docetaxel increased the level of survivin gene expression. Under a fluorescence microscope, the green fluorescence intensity (survivin MB-FITC) was stronger in the cells treated with either EGF or docetaxel compared with untreated control whereas the fluorescent signal for GAPDH molecular beacon (Cy3, red) was relatively consistent (Fig. 4*A*). We further used FACScan analysis to determine the mean fluorescence intensity in each cell population. Consistent with our observation with the fluorescence microscopy, we detected higher levels of fluorescent signal in EGF-treated cells compared with the untreated group in breast cancer cells (Fig. 4*B*). The relative level of survivin mRNA could be quantified from the FACScan data using the fluorescence unit of *GAPDH* gene as an internal control. We found that EGF treatment induced ~1.5-fold increases in the level of survivin gene expression in breast cancer cells.

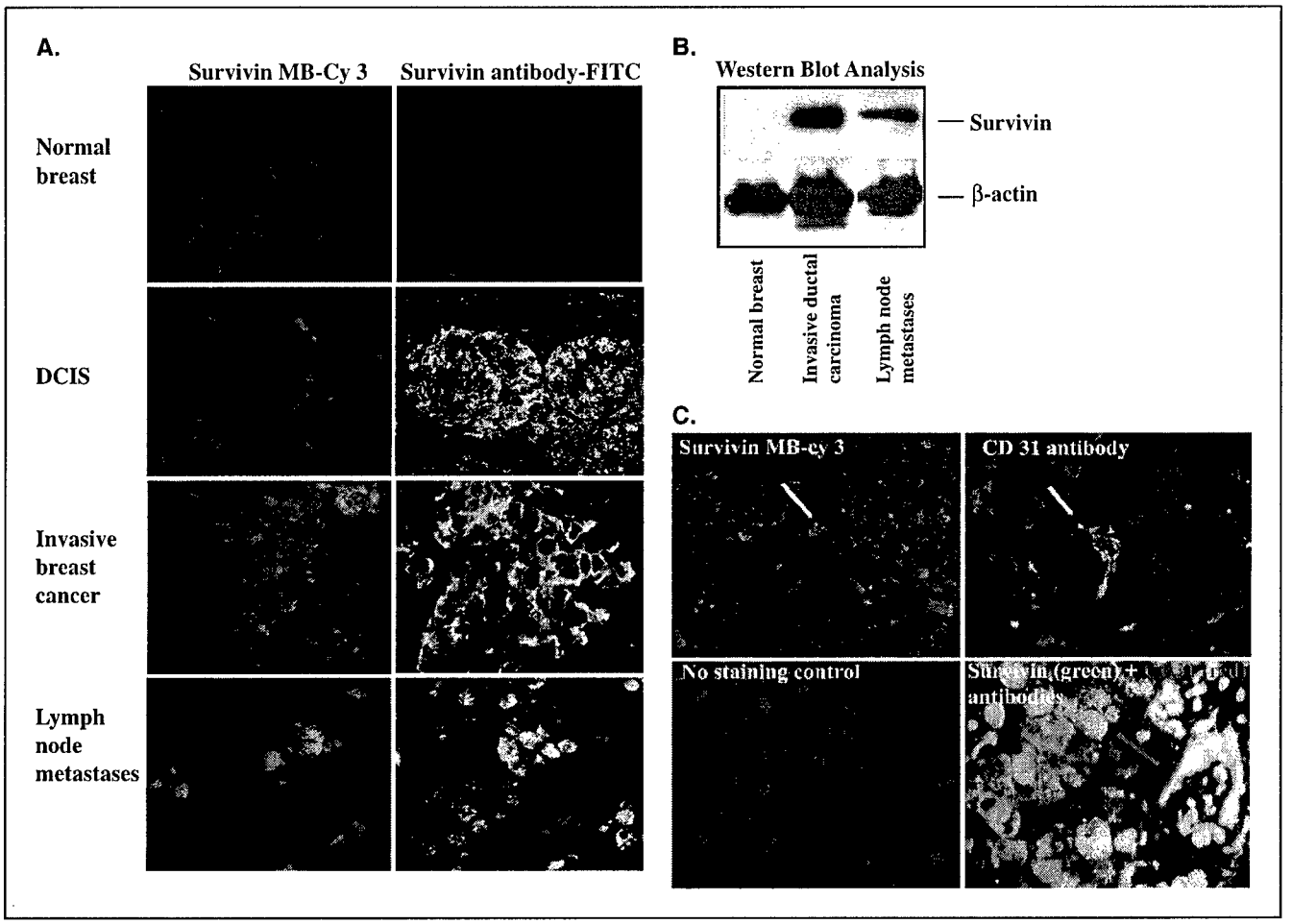

Figure 3. Detection of survivin gene expression on frozen tissue sections obtained from patients with breast cancer. Expression of survivin gene was detected in different stages of breast cancer tissues. Frozen tissue sections were fixed with acetone and incubated with survivin MB-Cy3. The sections were counterstained with Hoechst 33342 (*blue nuclei*). Survivin-expressing cells (*red*) were found in all stages of breast cancer tissues including DCIS, invasive carcinoma, and lymph node metastases, but not found in normal breast tissues. Different sections from the same tissues were also stained with a survivin antibody to confirm the presence of survivin positive cells (*green*). *B*, Western blot analysis showed a high level of survivin protein (16.5 kDa) in primary breast cancer and lymph node with metastases but not in normal breast tissues. *C*, detection of survivin gene expression in breast cancer and tumor endothelial cells in breast cancer tissues using double-labeling survivin MB-Cy3 with an antibody to CD31. Expression of survivin mRNA was labeled by survivin MB-Cy3 (*red*) and tumor endothelial cells were labeled with an anti-CD31 antibody (*green, yellow arrow*). Another tissue section was double-labeled with goat anti-human survivin (FITC, *green*) and mouse anti-human CD31 antibodies (*red*). All sections were counterstained with Hoechst 33342 (*blue*). *Red arrow,* tumor endothelial cells expressed both survivin and CD31 (*orange*).

In addition to the detection of levels of up-regulated genes, we examined the feasibility of quantifying the relative level of down-regulated gene expression. It has been shown that overexpression of *p53* gene decreases the expression of survivin gene (22). We transduced the tumor cells with Adp53 vector or control vector Adcmv for 24 hours and then delivered survivin or GAPDH molecular beacons into the transduced cells. Using FACScan analysis, we found that the relative fluorescence was decreased ~2-fold in Adp53 vector–transduced cells compared with the untreated or empty Adcmv vector control group (Fig. 4*C*). The ability of molecular beacons to detect a decreased level of gene expression suggests that the fluorescent signals detected intracellularly after molecular beacon transfection are not from nonspecific degradation of the molecular beacons because the same amount of survivin and GAPDH molecular beacons is delivered into Adp53 and control vector–transduced cells. The results from real-time reverse transcription-PCR (RT-PCR) further confirmed that EGF increased the transcription of survivin gene and overexpression of the *p53* gene decreased the level of survivin mRNA (Fig. 4*D*).

Although detection of the level of gene expression by FACScan could accurately measure the fluorescence intensity in individual cells as well as in cell populations, the procedure for FACScan is time-consuming and does not easily detect changes of gene expression in real time in the same cell population. To develop a high-throughput method for monitoring the changes of gene expression in real time in viable cells, we examined the feasibility of detecting levels of gene expression in cells cultured in 96-well plates using the molecular beacon-transfection approach. Breast cancer cells were plated in 96-well plates and transfected with a mixture of survivin and GAPDH molecular beacons for 3 hours. After adding EGF or docetaxel, the fluorescence units were

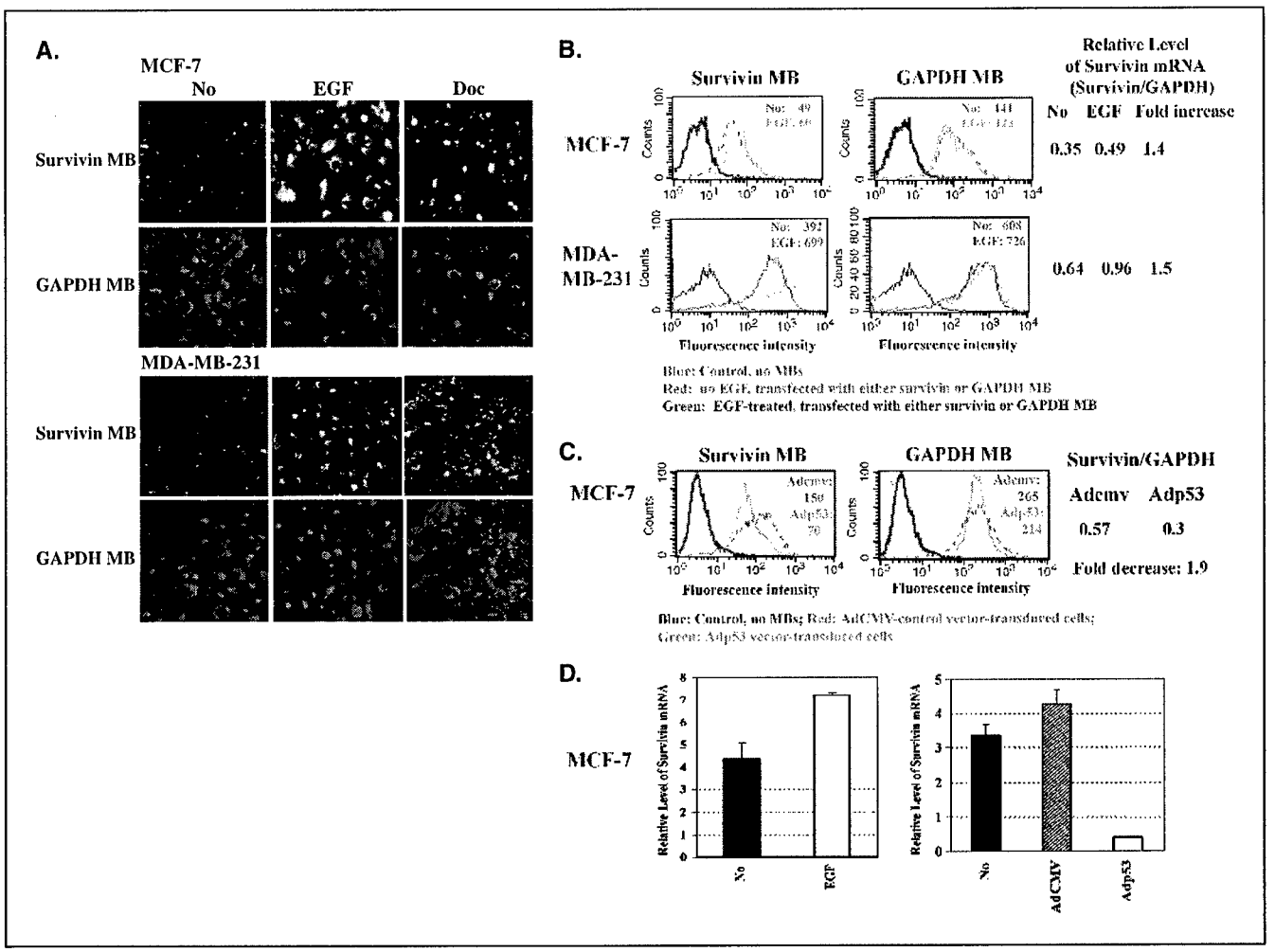

Figure 4. Detection of the levels of survivin gene expression in viable cells using survivin molecular beacon. *A*, survivin MB-FITC produced green fluorescence in cytoplasm of breast cancer cells after transfecting into viable cells. Treatment of the cancer cells with EGF for 1 hour or docetaxel (Doc) for 24 hours increased the fluorescence intensity in the cells. The fluorescence intensity generated by GAPDH MB-Cy3, which was cotransfected with the survivin molecular beacon, was relatively consistent in the cells. *B* and *C*, The level of survivin or GAPDH mRNA in molecular beacon-transfected cells could be measured by FACScan analysis to determine the mean fluorescence unit for each sample. The level of survivin mRNA was increased in EGF-stimulated cancer cell lines (survivin molecular beacon, *green line*), whereas there was no change in the fluorescence units detected in GAPDH molecular beacon–transfected cells (GAPDH molecular beacon, *green line*). On the other hand, the level of survivin mRNA decreased in Adp53 vector- (*green line*) but not in Adcmv vector–transduced cells (*red dotted line*). Numbers in the figure represent the mean fluorescence intensity for each group. Relative levels of survivin mRNA were calculated from the ratio of mean fluorescence intensities of survivin molecular beacon and GAPDH molecular beacon. Real-time RT-PCR analysis showed that the level of survivin mRNA was increased by EGF treatment but decreased after overexpression of *p53*. The numbers in the figure represent the mean numbers from three repeat samples. The relative level of survivin gene expression was calculated as a ratio of the quantity of survivin and GAPDH PCR products.

measured at different time points using a fluorescence microplate reader. We found that EGF-induced up-regulation of survivin gene expression occurred as early as 15 minutes after the treatment and lasted for >3 hours (Fig. 5*A*). There were 2.3 (MCF-7)- to 2.8 (MDA-MB-231)-fold increases in the relative levels of survivin mRNA after EGF treatment. We also examined the level of survivin protein using Western blot analysis and further confirmed that the level of survivin protein increased after EGF treatment (Fig. 5*A*).

For real-time detection of the level of gene expression in viable cells, it is important to determine how long the molecular beacon probes will stay in the cells and still be able to produce fluorescent signals that reflect the relative level of the gene expression. It has been shown that the chemotherapy drug docetaxel increases in the level of survivin gene expression as early as 4 hours after the treatment (21). We examined the level of real-time survivin gene expression in molecular beacon–transfected cells after docetaxel treatment from 0 to 48 hours. We found that the level of survivin mRNA was increased at 5 hours and reached higher levels 24 and 48 hours after treatment (Fig. 5*B*). The relative level of survivin mRNA is ~1.5-fold higher in docetaxel-treated cells than control cells and the difference detected 48 hours after docetaxel treatment is statistically significant (Student's t test, $P < 0.05$ for both MCF-7 and MDA-MB-231 cell lines). We also found a similar increase in the level of survivin mRNA detected by real-time RT-PCR compared with survivin molecular beacon detection, and the level of increase in survivin protein after docetaxel treatment (Fig. 5*B*, *inset* is real-time RT-PCR result).

One of the important issues to be addressed in developing an oligo-based approach for detecting gene expression in viable cells is whether the binding of the molecular beacon probes to their target RNA leads to degradation of the mRNA by RNase H, which may affect the level of target mRNA (23). To answer this question, we transfected breast cancer cells with either survivin molecular beacon or control GAPDH molecular beacon for 24 hours and then examined the level of survivin protein by Western blot analysis. We found that compared with cells transfected with a nonspecific GAPDH molecular beacon, the presence of the survivin molecular beacon in the cells did not reduce the level of survivin protein (Fig. 5*C*).

Discussion

We have developed a novel molecular beacon-based molecular imaging approach that allows identification of tumor cells expressing specific marker genes. Because molecular beacon is highly specific in detecting target mRNAs, and molecular beacons targeting various genes can be labeled with different fluorescent dye molecules and delivered into single cells, expression of several tumor marker genes in a single cell can be analyzed at the same time. Human cancers contain heterogeneous cell populations with various genetic changes (24). Simultaneous detection of overexpression of several tumor marker genes, especially when a single cell expresses more than one marker gene, may have a high predicative value for identifying cancer cells and therefore increase the sensitivity and specificity of cancer detection. Using molecular beacons targeting survivin and cyclin D1 mRNAs, we showed that delivery of a mixture of survivin and cyclin D1 molecular beacons into fixed cells produced fluorescent signals in breast cancer cells but not in normal breast cells. Interestingly, the fluorescence intensities in the cells correlated well with the level of the gene expression in different tumor cell lines. Previous methods for detecting gene expression *in situ* were not quantitative because the signals were amplified by either the presence of multiple fluorescent dye labeled nucleotides in an oligonucleotide probe or amplification of the signals with secondary antibodies to labeled nucleotides. Because each molecular beacon has only one fluorophore and unbound molecular beacons do not fluoresce, the fluorescence intensity generated by hybridization of the molecular beacon with a specific mRNA should reflect more accurately the level of the mRNA expressed in the cells.

At present, molecular beacon technology has been mainly used in various applications *in vitro*, which were done in solutions with defined molecular beacon-target conditions. Although previous studies showed the feasibility of detecting mRNAs and monitoring the transportation of RNAs in cells, the procedure for delivery of the molecular beacons through microinjection or by liposome delivery has made it difficult to apply this technology into broad research areas or into a routine clinical procedure (9–12). A recent study showed that it is feasible to transfect a molecular beacon into living cells to detect doxorubicin-induced activation of p21 gene expression (13).

We developed this molecular beacon-based procedure for the detection of gene expression in viable cells. We showed that transfecting survivin molecular beacon into cells produces a strong fluorescent signal in survivin-expressing tumor cells and the level of survivin gene expression can be monitored real time in cells either by FACScan or by using a fluorescence microplate reader. Using these methods, we detected an increase in the level of survivin gene expression following EGF and docetaxel treatment. Although we used GAPDH molecular beacon as an internal control for our experiments, simultaneous detection of survivin and *GAPDH* gene expression real time in viable cells indicates that it is feasible to monitor the levels of expression of several genes in the same cell population using molecular beacons labeled with different fluorophores.

Quantitative measurement of mRNA levels by molecular beacons is very important for the future use of this technology for cancer cell detection because many tumor marker genes are not unique to cancer cells and the difference between normal and cancer cells can be only the level of gene expression. Although we used two molecular beacons to detect the expression of tumor marker genes, a proof of principle from this study will lead to the use of more molecular beacons with multiple dye molecules to analyze the expression of several tumor genes. In addition, because only a small amount of abnormal cells are present in a large amount of normal cell background in clinical samples, there is a clear advantage of direct fluorescence imaging of individual cells expressing tumor marker genes for early detection of cancer cells compared with conventional RT-PCR to amplify the expression of tumor marker genes from isolated total RNA, which may be difficult to detect the differences in the level of gene expression in a few cancer cells over the normal background.

Current methods for the identification and classification of cancer cells from clinical samples rely on examining the morphology of the cells or immunostaining with antibodies for tumor-related protein markers. Although the *in situ* hybridization using labeled linear probes has been used to detect gene expression in tissue sections, it is very time-consuming and

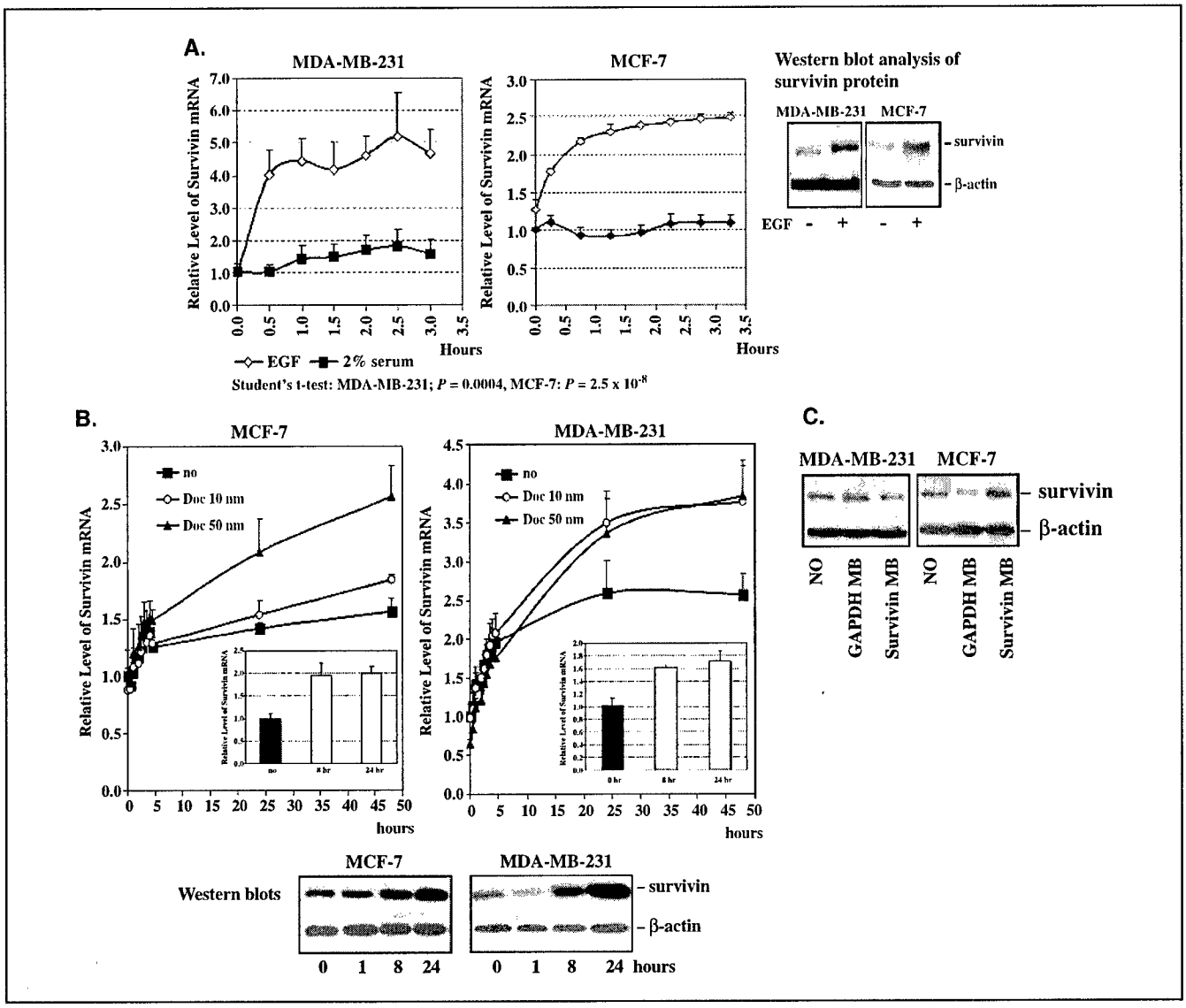

Figure 5. Real-time monitoring the level of survivin gene expression in breast cancer cells. Cells cultured in 96-well plates were transfected with a mixture of survivin MB-FITC and GAPDH MB-Cy3 and then added human EGF or docetaxel. The fluorescence intensity was measured at different time points following treatment using a fluorescence microplate reader. Each point in the curve is a ratio of the mean fluorescence unit of survivin molecular beacon (FITC, Ex/Em 480/530) and mean fluorescence unit of GAPDH molecular beacon (Cy3, Ex/Em 530/590) from four repeat samples. Similar results were obtained from three independent studies. *A*, EGF treatment significantly increased the level of survivin mRNA (Student's *t* test for all time points, $P < 0.0005$). Western blotting further showed that EGF increased the level of survivin protein. *B*, docetaxel treatment increased the level of survivin gene expression. Significant increases in the level of survivin mRNA were seen 24 to 48 hours following the treatment (Student's *t* test, $P < 0.05$). The levels of survivin gene expression after treatment were also examined by real-time RT-PCR (*inset*). Western blot analysis showed up-regulation of survivin protein by docetaxel. *C*, transfection of survivin or control GAPDH molecular beacon into viable cells did not significantly decrease the level of survivin protein as determined by Western blot analysis of cell lysates after transfected with either survivin molecular beacon or GAPDH molecular beacon for 24 hours.

usually accompanied by a high background because unbound probes also produce fluorescent signals. In our study, we found that molecular beacons could be used to detect the expression of genes on frozen tissue sections. The procedure is very simple and results can be examined within 30 to 60 minutes without the extensive staining and washing steps. Demonstration of the feasibility of combining the molecular beacon and immunofluorescence approaches to detect the expression of tumor marker genes and proteins *in situ* in the same cell population makes its potential application in pathologic diagnosis of human cancers more appealing. It is possible that the level of gene expression detected by molecular beacon-fluorescence imaging in clinical samples with intact tumor cells, such as fine-needle aspirates and exfoliated cells in body fluids is more quantitative than that detected in cancer cells on frozen tissue sections because most cells in tissue sections have been cut through and lost part of their cellular components.

One concern in the delivery of unmodified molecular beacons to viable cells is that the molecular beacons may be digested by nucleases in the cells or nonspecific interaction between molecular beacons, and cellular proteins may open up the stem of the molecular beacons, resulting in nonspecific fluorescence. However, our results showed that the fluorescence intensity detected by either FACScan or microplate reader correlated well

with the level of survivin mRNA in the tumor cells. Because a similar level of the molecular beacons was delivered into the tumor cells, it seemed that increases in the fluorescence intensity in EGF- and docetaxel-treated cells or a decrease in *p53*-expressing cells were not due to nonspecific degradation of the molecular beacons.

In this study, we showed that molecular beacon imaging of tumor cells is a simple and specific approach for the detection of breast cancer cells. This study is the first to apply state-of-the art molecular beacon–based methodology for cancer cell detection and for real-time monitoring the level of expression of tumor marker genes in viable cells. Based on this study, high-throughput assays for measuring the expression of multiple genes critical for drug response can be developed for screening cancer drugs that target specific molecules or pathways in cancer cells. To increase the specificity of molecular beacon detection, the molecular beacons can be further modified to make them resistant to nuclease or RNase H, such as by using 2′-*O*-methyl molecular beacon probes (12).

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